OVEREXPRESSION AND CHARACTERISATION OF HETEROLOGOUS ESTERASES FROM A METAGENOMIC LIBRARY

By

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BSc (Hons) Microbiology

Submitted in fulfilment of the academic requirements for the degree of Master of Science in School of Molecular and Cell biology

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Preface

The experimental work described in this dissertation was carried out in the Discipline of Microbiology and Biotechnology, School of Molecular and Cell biology, University of Witwatersrand, Johannesburg, from July 2014 to September 2015 under the supervision of Dr Karl Rumbold.

These studies represent original work by the author and have not otherwise been submitted in any form to another University. Where use has been made of the work by other authors it has been duly acknowledged in the text.

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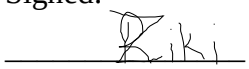

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Acknowledgements

Firstly, my sincere gratitude goes to my supervisors Prof K. Rumbold and Dr M.L Bode for taking me in as their student. They have been great mentors to me and many thanks for encouraging this research and allowing me to grow as a research scientist. I feel honoured having worked with you guys and thank you for the great advice and encouragement. None of this would have been possible without you guys.

Great appreciation also goes to Prof Dean for the biocatalysis input and a chance to do some chemistry work and the use of the organic chemistry research group machines. Also a thank you for the chance to do presentations with this group and they helped build up my confidence and brought a lot of knowledge on the synthesis of chemical compounds.

A big thank you to the Industrial Microbiology and Biotechnology members for all the help in molecular biology techniques and the general laboratory activities. I felt at home with you guys and I had a good time working with you all.

My appreciation also goes to Prof C. Rey and her students for the use of the machinery and the advice during the course of the research. You guys are great and keep up the good work.

A special thank you to the University of the Witwatersrand for the funding. I greatly appreciate and will never forget how this changed my life.

To my family and friends, thank you for the support throughout the year. Special mentioning goes to my darling husband Tichaona Mapengo. I do not know what I would have done without you in my life and you are my pillar of strength. To my son Ryan Mapengo, I always looked forward to seeing the cute face every day after school. It kept me going through and through.

To my mother, I say thank you for valuing my education and making sure that I get the best so that I can do my best at school. This thesis is largely dedicated to my late Dad. I am sure you are smiling from the heavens because this has always been your dream for me. Thank you and may your soul continue to rest in peace.

My friend Yvonne, thank you for being a great sister and for the encouragement to follow my dreams. To my aunt Sarah Ziki, thank you for being there for me every day being more of a mother to me and my son and encouraging me to keep up the hard work.

Finally to all my in-laws, thank you for valuing education and for the support and the desire to see me succeed beyond comprehension.

Abstract

Esterases are hydrolytic enzymes that have many industrial applications. They are used in food, pharmaceutical, pulp and paper, cosmetics, biofuels and many other industries. This gives research of these enzymes major importance. Esterase genes received from CSIR Biosciences were cloned in *E. coli* DH5α cells. The plasmids carrying these genes were pET20b(+) for genes named Est1, Est2, Est3, Est4, Est5, Est6, Est7, Est8, Est9, Est10, Est12, Est13, Est14 and pET28a(+) for Est11. These plasmids were extracted from the cloning host and transformed into the expression host which was *E. coli* BL21. The cells were then induced for expression and the presence of the protein bands representing the products of expression were confirmed by running the crude enzyme extract on SDS-Page. The enzyme extracts were tested for activity using pNp-acetate. All 14 esterases were active and they were characterised in terms of pH optima, temperature optima and kinetics. The enzymes showed a pH range of 6.0 to 9.0 and temperature range of 30°C to 50°C. The enzymes were investigated for substrate specificity and they showed a greater preference for short acyl chain substrates over long acyl chain substrates. Further testing was done for activity of the enzymes using α-naphthylbutyrate and naphthol AS-D chloroacetate alongside lipases. A total of 87 enzymes were tested using these colorimetric assays and 36 of the enzymes were found to be active including all 14 esterases. These 36 enzymes were tested for use in enzymatic resolution of three different chemical compounds available as racemic mixtures. No success was observed for two of the compounds but one of them showed some enantioselectivity. This research will be furthered on at large scale to allow continued synthesis of potential HIV-1 protease inhibitors.

List of abbreviations

APS	ammonium persulphate
dH_2O	distilled water
EDTA	ethylene diamine tetraacetic acid
IPTG	isopropyl β-D-1 thiogalactopyranoside
LB	luria Bertani
SDS-PAGE	sodium dodecyl sulfate-polyacrylamide gel electrophoresis
TEMED	N,N,N',N'- Tetramethylethylenediamine
SOC	super optimal broth
TE buffer	tris-EDTA buffer
DNA	deoxyribonucleic acid
GuHCL	Guanidium chloride
PAF-AH	human platelet activating factor acetylhydrolase
HSL	hormone sensitive lipase
HPLC	high performance liquid chromatography
FAE	feruloyl acid esterase
OPTIBIOCAT	optimised esterase biocatalyst for cost-effective industrial production
GE	glucuronyl esterases
HIV	human immuno virus
AIDS	acquired immune deficiency syndrome
PADAM	passerini amine deprotection acyl migration
MCR	multi component reaction

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CHAPTER 1: MOTIVATION AND BACKGROUND

1.1 Statement of the problem

Due to the HIV/AIDS pandemic, there has been much need to develop drugs that reduce or inhibit the action of the virus worldwide. The School of Chemistry at the University of the Witwatersrand has on-going research using branched isocyanides in the construction of possible inhibitors of HIV-1 protease. The compounds are prepared by means of the Passerini-amine deprotection acyl migration (PADAM) sequence. The Passerini multi-component reaction (MCR) couples an aldehyde, a carboxylic acid and an isocyanide to yield a product with a new stereogenic centre and both ester and amide functions (Gravestock *et al*., 2012). The attempted separation of a diastereomeric mixture of these compounds using selective enzymatic hydrolysis of one diastereomer has been a problem as the use of lipases and other enzymes yielded little if any of the desired hydrolysis products. Therefore this has prompted the need to try out the hydrolysis using esterases to see if there will be any catalysis in this reaction. The expression and characterisation of the enzymes gives a better understanding of the optimum conditions that the enzyme can work in as it is utilised in various industries.

1.2 Justification

Esterases are isolated from many different sources. Microorganisms such as *Dickeya dadantii, Fusarium oxysporum, Burkholderia multivoruns, Aureobasidium pulpulans, Aspergillus niger, Aspergillus flavipes, Aspergillus awamori* and *Aspergillus oryzea,* are known to secrete carboxylic ester hydrolases as well, especially feruloyl acid esterases (Kashima *et al*., 1998). The properties of these esterases do not always meet the requirements for the given applications thereby limiting their usefulness. The applications of esterases cover a broad spectrum in industry. These industries include the food and food additives industry, pharmaceutical industry for the synthesis of bioactive chemicals, cosmetic industry for flavour and fragrance precursor production, the animal feeds and feed additives industry and the paper and pulp industry as bleaching agents (Panda and Gowrishankar, 2005). Because esterases have a broad range of applications much research interest has been sparked which is evidenced by the discovery of many feruloyl esterases from microorganisms over the past few years.

Termite species digest lignocellulose-based materials very efficiently with the support of microbial symbionts associated with their hindgut (Wheeler *et* al., 2009*;* Watanabe *et* al., 1998). This section serves as a metabolic engine for the breakdown of many compounds. Esterases have been implicated to play a role in the termites' physiology and metabolism (Davis *et al*., 1995). With the use of the metagenomic approach, several esterases were isolated from the termite enteric flora and from water from mine dumps in the Eastrand, South Africa (Rashamuse *et al*., 2013). In this study the enzymes were subsequently expressed in a host, *E.coli* BL21 (DE3), then the enzymes were characterised to help understand the optimum conditions for esterase hydrolysis and they were used in their crude extract form for the separation of various diastereomeric and enantiomeric mixtures to synthesise pure compounds. Enzymes are highly enantioselective and this gives them major importance in the chemical synthesis of optically pure compounds.

1.3 Aims and objectives

The overall aim of this study was to express and characterise the 14 esterases isolated from various metagenomic libraries and use them for the synthesis of organic compounds that can be used as HIV-1 protease inhibitors.

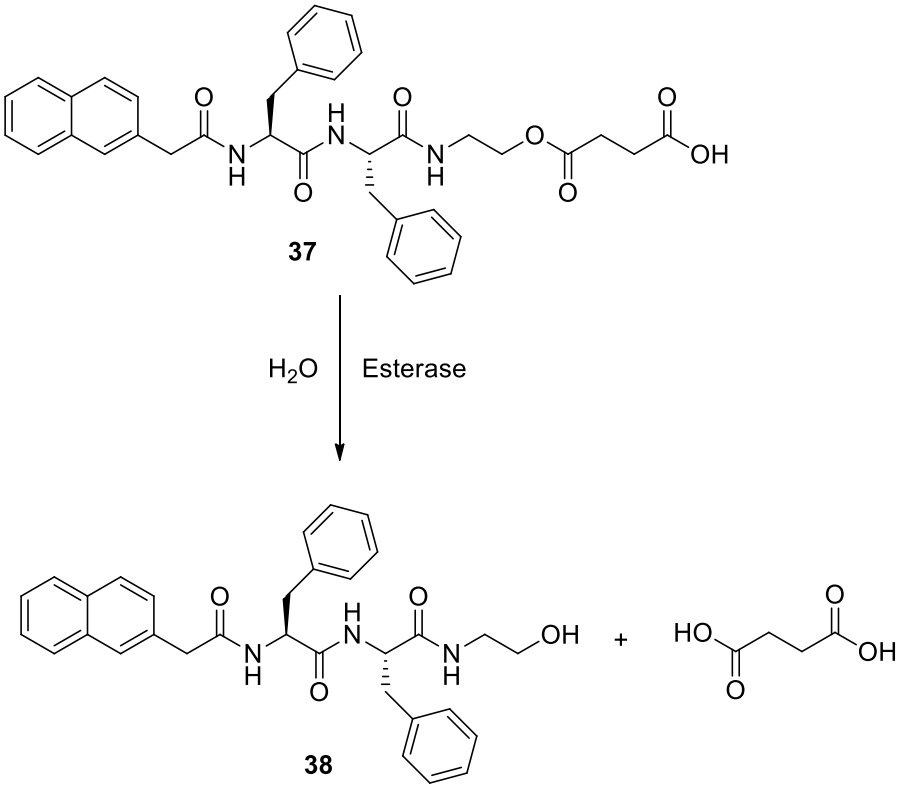
The specific objectives of this study are:

1. To isolate the plasmids carrying the esterase gene from the cloning host
2. To transform the expression host.
3. To determine expression profiles of several esterases from various sources.
4. To functionally characterise the esterases.
5. To use the crude extracts of the enzymes in organic reactions.

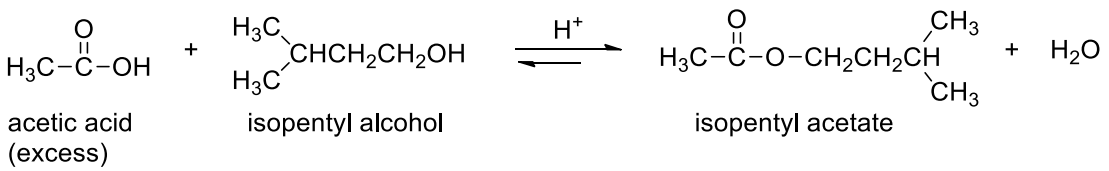
CHAPTER 2: LITERATURE REVIEW

2.1 Introduction

Esterases are hydrolytic enzymes that catalyse reactions leading to the formation or cleavage of ester bonds (Bornscheuer *et al*., 2002). They hydrolyse esters into an acid and an alcohol but the different kinds of esterases have different substrate specificities and they vary in protein structure and biological function (Panda *et al*., 2005). The reaction below (Scheme 2.1) shows the hydrolysis of an ester into an acid and an alcohol, a reaction that is catalysed by esterases. Certain organic substrates are suitable for esterases and lipases to catalyse esterification (Scheme 2.2); and interesterification and various hydrolysis reactions that lead to synthesis of alcohols and acids or formation of esters (Topakas *et al*., 2004).



Scheme 2.1: Hydrolysis reaction example using an esterase enzyme. (Banerjee *et al*, 2009)



Scheme 2.2: Example of an esterification reaction. (www.dbooth.net/preparation of an ester)

As esterases represent a wide group of hydrolases, they are an important group of enzymes. Hydrolases are grouped into two categories called lipases and carboxylesterases. Lipases are also known as triacylglycerol acylhydrolases (EC: 3.1.1.3) and carboxylesterases are also known as carboxylester hydrolases (EC: 3.1.1.1) (Arpigny and Jaeger, 1999). In the hydrolysis of triacylglycerols by lipases, monoacylglycerols, glycerol and free fatty acids are formed (Jaeger *et al*., 1999). Carboxylesterases catalyse the hydrolysis of small molecules containing esters and the molecules are partly soluble in water, unlike lipases which work on molecules that are insoluble in water and are long-chain triacylglycerides (Arpigny and Jaeger, 1999).

Esterases are purified from various sources that include microorganisms, plants and even animals. All microorganisms including bacteria, actinomycetes and fungi produce esterases either naturally or when chemically induced. The cost of producing microbial enzymes is cheap which makes them attractive sources and also their growth is fast and maintenance is cheaper. Esterases have also been isolated from the deep gold mines in South Africa (Abbai *et al*., 2011). Metagenomic screening also provides information on esterases from metagenomic libraries (Kim *et al*., 2005). Contaminated oil and garbage in cities has also been studied and esterases were reported to be found in these places (Bhardwaj *et al*., 2012). Termites also were studied and esterases were isolated from their hindguts (Rashamuse *et al*., 2007).

Carboxylic acid esterases have various subclasses that include Feruloyl acid esterases (FAEs) that mainly catalyse reactions on ester bonds between hydroxycinnamic acids and plant cell wall sugars (Vafiadi *et al*., 2006a). Studies show the purification of these enzymes from fungi and bacteria (Rumbold *et al*., 2003), and recently this enzyme was purified from human intestinal bacteria (Wang *et al*., 2001). Four groups exist within the feruloyl esterase subclass and they are named from A to D. These groups differ in terms of the specificity that the enzymes have for diferulates and monoferulates and also in their amino acid sequences (Wang *et al*., 2001). Esterases have various industrial functions making their study important. They are used in the pulp and paper industry, food, pharmaceutical, cosmetics, detergents and biofuel industries. The synthesis of flavour esters in food manufacturing and fat and oil manufacture as well as production of pure chemicals from racemic mixtures in the pharmaceutical industries can be achieved by utilising esterases (Molinari *et al*, 1996).

2.2. Structure of Esterases

The three-dimensional structures of lipases and esterases have a characteristic α/β hydrolase fold, which has a definite order of α-helices and β sheets (Fig 2.1). The α/β fold has been widely described as 8-stranded mainly parallel β sheets surrounded on both sides by α-helices. The identification of the α/β hydrolase fold was done by comparison of 5 hydrolytic enzymes that had different catalytic functions. This family of α/β hydrolases is one of the most versatile families as it contains lipases, esterases, epoxide hydrolases, proteases, peroxidases and dehalogenases (Nardini and Dijkastra, 1999). The catalytic part of the enzymes is made up of Ser-Asp-His and the Asp is replaced by Glu in lipases. There is also usually a common sequence of Gly-x-Ser-x-Gly found around the active site. Esterases contain a motif that is made up of Gly-x-x-Leu motif as well as high similarity to class C β-lactamases (Wei *et al*., 1995). Microbial carboxylesterases have major importance because they have a wide range of substrates that they work on due to the high structural differences and conformations and they are highly active and stable in organic solvents (Buchholz *et al*., 2005).

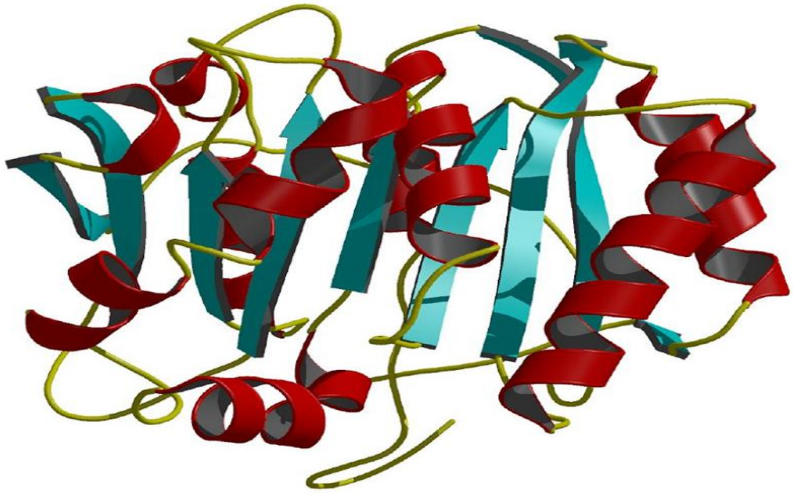


Figure 2.1: Feruloyl esterase 1A (CE1), *Clostridium thermocellum* (*nzytech genes and enzymes website*).

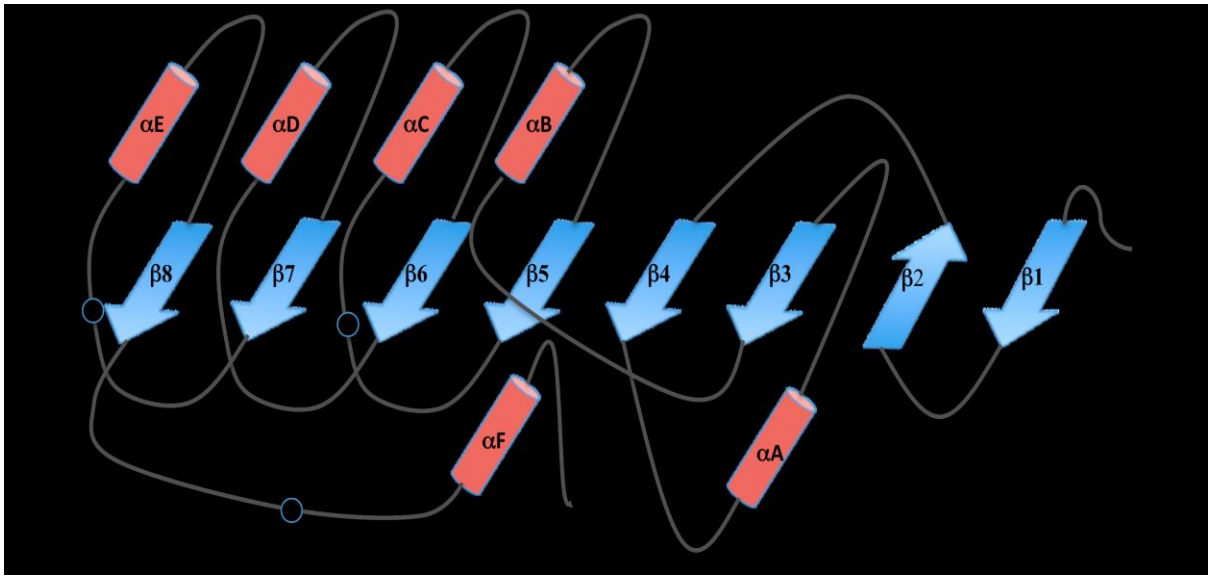


Figure 2.2: Schematic representation of α/β folds in esterases. (Bornscheuer U.T, 2002)

2.3. Sources

Over the years, studies have been done to characterise esterases from different sources that include both thermophilic and mesophilic niches (Vecchio *et al*., 2009). Despite the enzyme source of origin, they show high conformational stability against temperature changes and even in the presence of urea and GuHCL. In a study done by Vecchio *et al* (2009) they determined this by means of circular dichroism, fluorescence and differential scanning. Esterases have been isolated from animals, plants and a wide range of microorganisms. In microorganisms, esterases have been isolated from all classes like bacteria, fungi and actinomycetes. They are produced either constitutively or by induction. Esterase production has been noted in organisms from cheese surfaces, city garbage that is even oil contaminated and marine squid (Gandolfi *et al*., 2000, Ranjitha *et al*., 2009, Bhardwaj *et al*., 2012).

New sources of esterases have become available as well, like metagenomic libraries using metagenomic screening techniques (Kim *et al*., 2005, Liu *et al*., 2008). Activated sludge metagenomes are also another source of esterases (Zhang *et al*., 2009). Techniques used to access the DNA content in a given environment are called metagenomics and they help researchers to access useful DNA content (Cowan *et al*., 2005). A number of DNA libraries have been developed successfully and this helps in the manufacture of antibiotics and new enzymes providing more applications in the medical and food industries (Gilliespie *et al*., 2005).These various applications of enzymes, including esterases have fuelled much research interest in enzymes evidenced by the discovery of new esterases from different sources,

especially microorganisms. Esterases isolated from microorganisms have many industrial uses including the synthesis of organophosphorus compounds (Wakai *et al*., 1990). For the synthesis of aromatic compounds such as isoamyl acetate, involvement of esterases isolated from *Saccharomyces cerevisiae* has been reported. Esterases isolated from *Acetobacter sp*. are used to produce ethyl acetate during vinegar fermentation (Kashima *et al*., 1998).

In a study done by Rashamuse *et al* in 2007, they used termite hindguts as a source of esterases. Various esterases have been isolated from insects as well and they have well defined biological functions. The functions include xenobiotic, lipid and acetylcholine metabolism. Because lignin has a highly esterified structure, generally esterases contribute to the depolymerisation of the lignin in small insects that feed on grass and wood, for example termites (Scharf and Tartar, 2008). Dead plant material degradation is made easier in termites because they are capable of symbiotic and endogenous cellulose digestion.

Bacteria and archaea isolated from a deep gold mine in South Africa were studied for novel esterases and it produced 2 estereolytic clones (Abbai et al, 2011). Some esterases have been isolated from crop juices like carrot and lettuce (Oxford, 1977). The diversity in the sources of esterases make them interesting for research as these enzymes show that they can withstand survival in different environments, making them applicable in many industries.

2.4. Classification

Arpigny and Jaeger in 1999 classified bacterial esterases and lipases mainly based on their primary amino acid sequences, biological properties that include how the enzymes are secreted and the specific folds that are found in the protein structures and also relationships within the enzyme families. There are 8 different families including both lipases and esterases named family I to family VIII. These families include both lipases and esterases and their comparisons show sequences that are remarkably similar although there are vast differences in the substrate specificities of the enzymes and their physiological functions (Lin *et al*., 2001). Family I is comprised of 6 other subfamilies that contain true lipases and the subfamilies are differentiated by differences in protein folds and sizes. Family II has lipases and esterases that all have a conserved sequence motif (Gly-Asp-Ser-Leu). In family III, there are lipases that have a conserved catalytic triad and they show 20% similarity to the Human-Platelet-activating factor acetylhydrolase (PAF-AH). Hormone-sensitive lipases (HSL) make up family IV. A conserved catalytic triad is also found in the remaining families but they are

distinguished by sequence similarities. Included in family V are epoxide hydrolases and haloperoxidases whilst family VI has eukaryotic acetylcholine esterases and class VII has intestinal /liver carboxylesterases. The final family VIII is comprised of *β*-lactamases.

2.5. Lipases and Esterases

Lipases and esterases are the two major classes of hydrolases with utmost importance (EC 3.1.1.1, triacylglycerol hydrolases) and 'true' esterases (EC 3.1.1.3, carboxyl ester hydrolases). Carboxylester hydrolases, commonly known as esterases are a large group of enzymes that are defined by their ability to hydrolyse carboxylic ester bonds. Lipases are lipolytic enzymes that constitute a special class of carboxylic esterases that hydrolyse long chain fatty acids (Ali *et al*., 2012). Several attempts have been made to differentiate lipases from esterases using various criteria. Ali *et al*., (2012), took into account the basic physicochemical criteria and they primarily distinguished these two groups of enzymes considering their chemical, anatomical and cellular nature. Their 3-D structures both show a characteristic *α*/*β* hydrolase fold (Bonscheuer 2002, Faiz *et al*., 2007).These enzymes also have a catalytic triad consisting of Ser-Asp-His (Kim *et al*., 2005).

Esterases hydrolyse short chain carboxylic acids of usually less than 12 carbons and lipases hydrolyse long and usually insoluble chains of triacylglycerides and secondary alcohols (Faiz *et al*., 2007). Lipases also show a pH dependent electrostatic signature where they have a negative potential at pH 8.0 whilst esterases show this trend at pH 6.0 (Bornscheuer, 2002). Lipases can also be distinguished from esterases by the interfacial activation phenomenon. In esterases kinetics follow a Michaelis-Menten theory. Higher activity is observed in lipases after subjection to minimum substrate concentration. This interfacial activation is due to the hydrophobic domain (lid) that covers the active site of lipases. Only if the active site is covered by minimum substrate concentration will the lid move apart leaving the active site accessible (Bornscheuer, 2006). Both esterases and lipases are stable and active in organic solvents, although this feature is more prominent in lipases.

2.6. Mechanism of action

Enzyme production requires suitable media that promote the enzyme synthesis, therefore sources of compounds which are essential in this synthesis should be considered as they affect the synthesis. For feruloyl acid esterases to be produced an inorganic source of nitrogen salts is required because the organic sources that are complex, for example extracts

from yeast, suppress enzyme production (Shin and Chen, 2005). Figure 3 shows the general diagrammatic representation of the mechanism of action followed by both esterases and lipases.

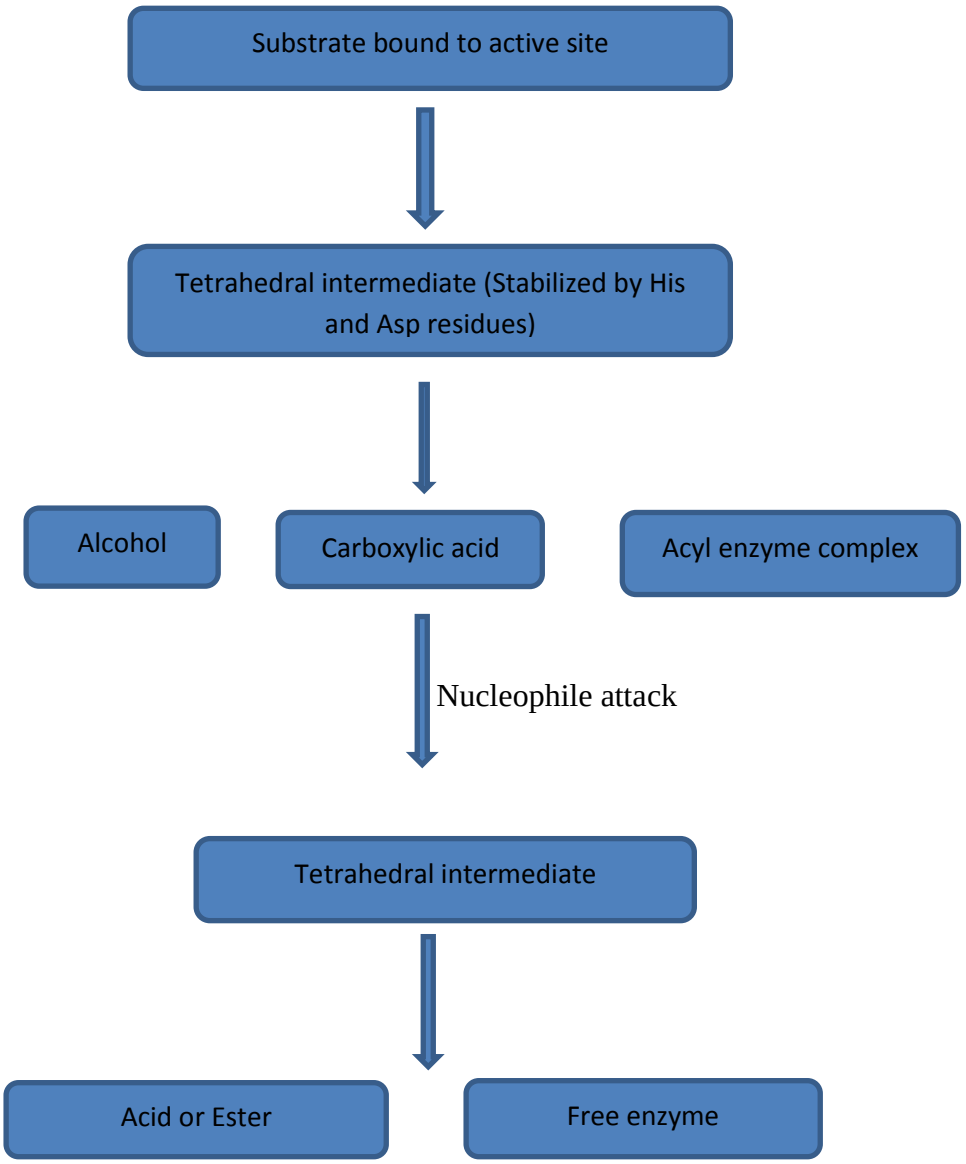


Figure 2.3: Representation of the mechanism of action by esterases and lipases on substrates.

2.7. Chemical synthesis

Esterification using enzymes provides an alternative to the poor selectivity that is normally experienced during chemical synthesis. The use of esterases in esterification is not commonly reported in literature (Vafiadi *et al*., 2005). Ternary water-organic solvent mixtures are potentially used as reaction systems during the esterification and transesterification of different cinnamic acids by feruloyl esterases (Vulfson *et al*., 1991). Hydrocarbon and short

chain alcohols in water make up these systems and thermodynamically they are very stable and visually they are transparent. They also serve as appropriate media during enzymatically catalysed reactions (Giuliani *et al*., 2001).

2.8. Substrate specificities

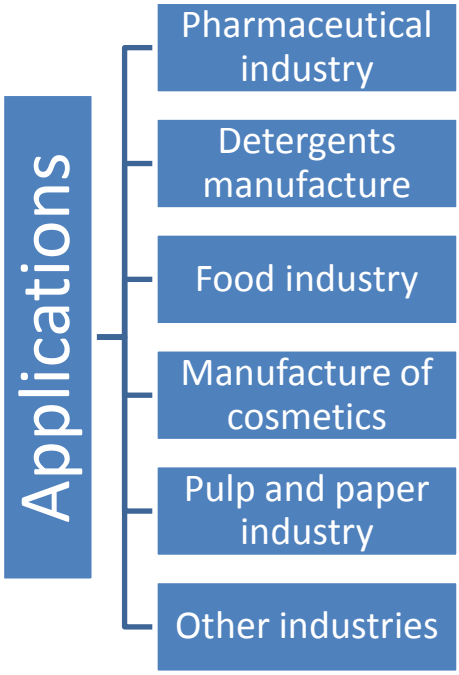
Positional isomers of feruloyl acid esterase substrates can be improved or synthesised enzymatically by acetylation of the starting compounds as described by Mastihubova and Biely (2010). The substrates include *p*-nitrophenyl, mono-*O*-ferulates, α-L-arabinofuranoside and *β*-D-xylopyranosides. Substrate specificities and differences in activities help differentiate feruloyl acid esterases and the activity is calculated by measuring the optical density using UV-spectrophotometric assays.

Differences in substrate specificities are also seen in esterases purified from different sources. Feruloyl esterases purified from a mesophilic source and from a thermophilic source have major differences in their active sites (Topakas *et al*., 2004). During comparison of esterases from these two different environments the phenyl-propionate structures of the substrates was maintained and alterations were done by substitutions to the aromatic ring. Methoxylated substrates were preferred by esterases from the fungus *Fusarium oxysporum* (FoFaeA) which was purified from a mesophilic source and *Sporotrichum thermophile* from a thermophilic source preferred hydroxylated substrates (Topakas *et al*., 2004). This indicates the differences in substrate preferences being influenced by the source of the esterase. Differences in affinities for 5-*O*- and 2-*O*- feruloylated, α-ι arabinofuranosyl residues is also seen in feruloyl esterases from different microorganisms such as *Aspergillus penicillium*, *Fusarium* and *Talaromyces* (Kroon *et al*., 2000).

Enzyme preparations are available commercially and they are usually prepared from *Humicola insolens*, *Thermomyces lanuginosus* and *Aspergillus niger* (Mastihubova *et al*, 2006). The hydrolytic action of feruloyl esterases on different methyl esters of cinnamic acids is parallel to their patterns of activity (Chen *et al*, 2011). A feruloyl esterase was isolated from *S. thermophile* by Vafiadi *et al*., (2006b), and it showed maximum activity on methyl ferulate compared to the other 26 substrates that were tested. This indicates the promising biocatalyst activities on feruloylation of aliphatic compounds.

2.9. Applications

Esterases have many industrial applications such as in the synthesis of optically pure compounds, antioxidants and perfumes, bioremediation, agriculture, and their use in the food and pharmaceutical industries. They are widely used as biocatalysts due to their ability to catalyse the hydrolysis of triacylglycerides in aqueous solutions and also the enantioselective synthesis of compounds in organic solutions. The ability to remain highly stable and active in organic solvents makes them very useful in biotechnology which involves high solvent use.



2.9.1. Pharmaceutical industry

Chiral drugs are of high importance in the treatment of certain diseases. Esterases have been involved in the synthesis of these drugs making them highly important in the pharmaceutical industry (Bornscheuer *et al*., 2002). An example is the carboxyl esterase that originated from *B. subtillis* which was used in the synthesis of naproxen (NP) a strong anti-inflammatory drug. The enzyme was used to selectively hydrolyse (*R*,*S*)-naproxen methylesters and only the (*S*)- acid was formed. FAEs have been heavily used in the tailored synthesis of pharmaceuticals as phenolic acid sugar esters have established antitumor activities and have potential to be used to create antimicrobial, antiviral and anti-inflammatory agents. The pig-liver esterase was also used in the synthesis of enantioselectively pure (*S*)-α-methylphenylalanine, (*S*)-α-methyltyroine and (*S*)-α-methyl-3,4-dihydroxylphenyalanine (Bjorkling *et al*., 1985). An esterase from *A. globiformis* also catalysed the synthesis of (+)-

trans-(1*R*,3*R*)-chrysanthemic acid, which is an important precursor for pyrethrin insecticides (Bornscheuer *et al*., 2002). Phenolic components of plant cell walls, for example *p*-coumaric acid, ferulic acid and *p*-hydroxybenzaldehyde inhibit growth of rumen microorganisms and these can be liberated from plant cell walls using ferulic acid esterases (Williamson *et al*., 1998).

2.9.2. Detergents manufacture

Esterases are major additives in industrial laundry and household detergents due to their ability to hydrolyse fats. They are mostly preferred as well because of their tolerance of pH and temperature changes as well as the ability to work in different solvents (Malcata, 1996).

2.9.3. Food industry

Feruloyl esterases are used in the production of cinnamic acid from plant cell walls which is a precursor in vanillin manufacture (Priefert *et al*., 2001). FAEs also catalyse the synthesis of sugar-phenolic esters (Topakas *et al*., 2004a, b). Ferulic acid is an aromatic antioxidant which can be extracted from maize fibre after wet milling and can be used as an important flavouring agent. Vanillin is also used extensively in the perfume industry and in the metal plating industry. Esterases are also used as a ripening agent for sucrose yield increase in the sugar cane industry (Wood and McCrae, 1996). FAEs were also used in a study done on a Japanese spirit,-*Awomari* production. It has a distinctive vanilla smell and esterases are used to produce that aroma (Makoto and Kanauchi, 1997). Esterase-lipolysed butterfat is relatively free of soapy/bitter flavours (Kanisawa *et al*., 1982). Pre-gastric esterases are used in the manufacture of feta cheese. Esterases can be used to alter fatty acid chains in upgrading palm oil to achieve properties similar to cocoa butter which is used in chocolate production.

2.9.4. Manufacture of Cosmetics

The cosmetics industry generally deals with the manufacture of products applied to the body to improve appearance. The European Union market together with Optimised esterase biocatalyst for cost effective industrial production (OTIBIOCAT) organisations promote research and innovation that replaces chemical processes being used in the cosmetics production with cost effective, energy efficient and eco-friendly bioconversions. The OPTIBIOCAT has specific objectives including the generation of novel FAEs and Glucuronyl esterases (GE) from microorganisms to be used in enzyme based reactions.

2.9.5. Pulp and paper industry

FAEs and acetylxylan esterases aid in the removal of linkages and substitutions during pulping, making the solubilisation of lignin-carbohydrate complexes easier (Mathew *et al*., 2004). Esterases are used to control stickies in paper mills. Studies revealed that stickies contain an ester-linkage that holds the building blocks together. Therefore, esterase-type enzymes control the stickiness by hydrolysing these bonds and the process improves paper cleanliness.

2.9.6. Other applications

Esterases are important again in the metabolism of plants and animals and also in their physiology (Sivakuraman and Mayo, 1991). They hydrolyse the endogenous substances as well as assuming roles in the intermediary metabolism or promotion of xenobiotic detoxification (Shen and Dowd, 1991).

In the breakdown of plant cell walls, many enzymes are secreted by the plants. Esterases break down the cross-linkings of polymer structures and sugars that are held together by phenolic rings (Hermoso *et al*., 2004). This breakdown is important as it aids in the opening of the cell wall to make plant material accessible by other glycosyl hydrolases. Complex highly branched polysaccharides (arabinoxylans, pectin), are the main components of the cell walls in plants. The complex polymer structures include highly branched polysaccharides, namely arabinoxylans and pectins. Covalent bonds hold together the polysaccharides with hydroxycinnamates through ester bonds, which complicates the structures further (Kroon *et al*., 1999). A battery of different enzymes is required to break down these structures and esterases are actively involved (Faulds and Williamson, 1994). Other enzymes involved are xylanases, pectinases and hemicellulase. More interest is given particularly to a subclass of carboxylesterases that contains feruloyl esterases and cinnamoyl esterases that hydrolyse the ester bonds between hydroxycinnamic acids and sugars (Bartolome *et al*., 1997).

The breakdown of bonds between hydroxycinnamic acids and sugars involves the acids undergoing oxidative coupling reactions that result in the formation of hydrodimers of acids and also the polymerisation of lignin (Feinberg *et al*., 1987). Therefore the hydroxycinnamic acids that aid in the build-up of plant cell walls are important to plants as they keep the wall together. This protects the plants from diseases or digestion by microorganisms (Saper, 1993).

In the maize industry corn cobs are produced as a by-product and they are mainly used as animal feeds (Torre *et al*., 2008). These agro-industrial wastes contain *p*-coumaric acid (*p*-CA) and ferulic acid (FA) which can be released if they are treated with the appropriate chemicals (Graf, 1992). The acids can later be transformed to antioxidants used in the preservation of food because they are capable of stopping peroxidation of fatty acids. This can be achieved by using esterases through reaction processes that are eco-sustainable (Faveri *et al*., 2007). In cereals the outer layers of the kernels are made up of hydroxycinnamic acids, mainly ferulic acid (Barron *et al*., 2006). Hydroxycinnamates and their derivatives were proved to be bioactive compounds which give them more importance in industrial biotechnology as they have antioxidant activity which is important for human health (Graf, 1992).

Grain germination in plants has been reported to be aided by feruloyl esterases (Sancho *et al*., 2001). Esterified ferulates are also hydrolysed by esterases during wound healing on plants as well as the process of stomata opening (Jones *et al*., 2003). This allows the phenolic acids to move around plant cells. Waste materials from cereal and plastic production can be degraded using esterases; this is an important process in the cleaning of the environment from industrial pollutants (Panda *et al*., 2005).

Feruloyl esterases work together with xylanases in hydrolysing ester-linked cell wall materials therefore this enzyme has importance in biomass degradation (Topakas *et al*., 2006). Xylans constitute a major part of wood and other agricultural residues and these polysaccharides are important in the industrial manufacture of animal feeds and the production of pulp and paper (Castanares *et al*., 1992). This means xylan-degrading enzymes are commercially important in selective modification of theses xylans. As esterases have many industrial applications, more research needs to be done on the factors that affect their activities (Mackenzie *et al*., 1987, Donnelly *et al*., 1988). The manufacture of perfumes also involves the use of esterases (Panda *et al*., 2005). Hydrolysis of fats by lipases and esterases is done during cheese manufacture and this enhances the flavour of the end-product (Fernanderz *et al*., 2000).

2.10. Esterases in biocatalysis

It is very important in biocatalysis to apply traditional enzyme screening techniques in the identification of microorganisms that are functional with certain substrates. Over the years

many carboxylesterases have been discovered and functionally screened considering their hydrolytic properties. Widespread research is being done on genome sequencing of thousands of microorganisms to provide large quantities of genome sequencing data that will aid in biocatalysis mining. Genome database data has so far helped in the cloning and characterisation of several esterases (Koseki *et al*., 2009). The chemical specificity of enzymatic transformations and also the ability of enzymes to work under mild conditions have made them preferred catalysts over chemicals (Fuciños *et al*., 2011). Biocatalysis is implemented in many industrial processes including the pulp and paper industry, food technology, agrochemicals and the synthesis of biologically active compounds for research and diagnostics.

Biocatalysis has quite a number of advantages that include thermal stability. Research on extreme thermophiles is important in this regard as they can be used as sources of temperature stable enzymes that do not denature in the presence of a number of chemical and physical agents (Antranikian *et al*., 2008). Enzymes also have very high enantioselectivity and regioselectivity and this makes them useful in the synthesis of optically pure compounds. When working with enzymes the solvent is often water making them important in 'green chemistry'.

In the pharmaceutical industry, enantiopure compounds are preferred over racemic mixtures and this brings in the need to separate the two enantiomers. In the synthesis of naproxen [*S*-(+)-2-(6-methoxy-2-naphthyl)propionic acid], an anti-inflammatory drug which is administered in its *S*-configuration, this compound is 150 times more active than *R*-naproxen (Jackson *et al*., 1995). *R*-naproxen was even shown to have unwanted side effects of gastro-intestinal disorders (Roszkowski *et al*., 1971). In enantioselectives separation it is important to optimise reaction conditions that prefer the hydrolysis of one enantiomer as this leads to a good yield of pure compounds.

CHAPTER 3: MATERIALS AND METHODS

3.1 Strains and Plasmids

The vector pET-20b (+) and pET-28a (+) were used for the cloning of the esterase genes and were again used as expression vectors of the enzymes. *E. coli* BL21 (DE3) was used as an expression host.

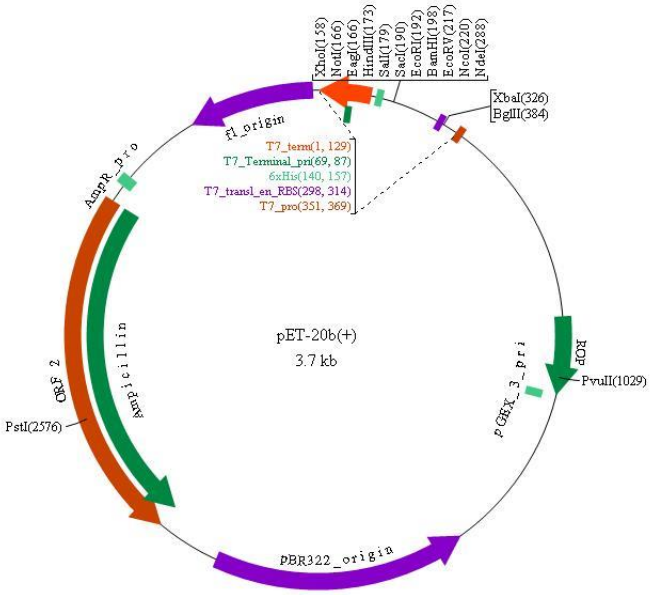


Figure 3.1: pET-20b (+) Plasmid. (www.biovisualtech.com)

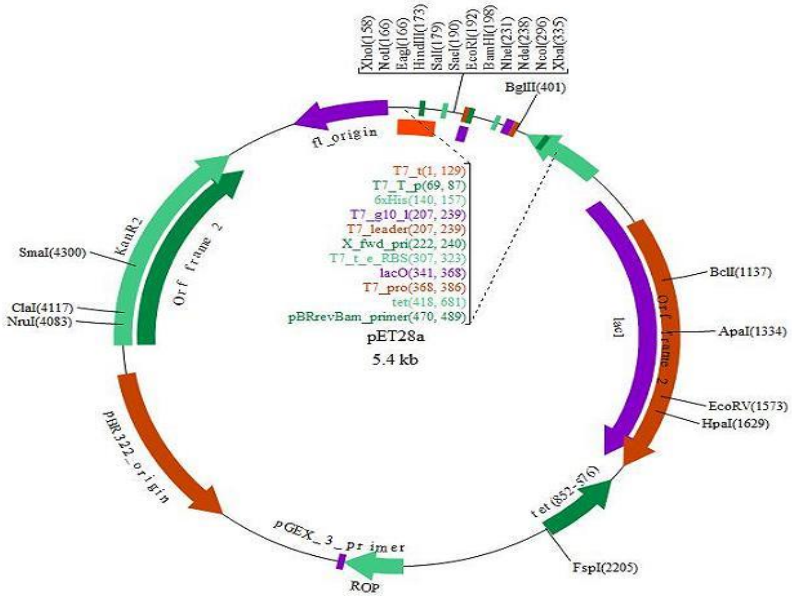


Figure 3.2: pET 28a (+) Plasmid. (www.biovisualtech.com)

3.2. General Reagents

The general materials and methods used throughout the research are outlined in this section of the thesis.

3.2.1. Ampicillin (100 mg/ml)

Stock solutions of the antibiotic ampicillin were prepared in advance and stored in 1ml aliquots at -20 degrees Celsius. Ampicillin sodium salt (1 g) was dissolved in distilled water and made up to a volume of 10 ml. For sterilization the solution was passed through a filter paper (0.22 µm) and finally aliquoted to 1 ml Eppendorf tubes and stored in the freezer.

3.2.2. Kanamycin (50 mg/ml)

Kanamycin disulfate salt (0.5 g) was dissolved in distilled water and volume was made up to 10 ml. The solution was sterilised by passing through a 0.22 µm filter paper and aliquots of 1 ml were put in Eppendorf tubes and stored at -20 degrees Celsius.

3.2.3. Luria Bertani (LB) Broth

E.coli cells used throughout this research were cultured in LB broth containing the desired antibiotic. The broth was prepared according to Sambrook and Russel (2001). Tryptone 1% (w/v), NaCL 1% (w/v) and yeast extract 0.5% (w/v) were dissolved in distilled water and filled up to the desired volume. The mixture was sterilized by autoclaving at 121 ℃ for 15 minutes. After autoclaving the solution was allowed to cool before adding the ampicillin or kanamycin antibiotics stock solutions to the desired final concentration which were 100 µg/ml and 50 µg/ml respectively.

3.2.4. Luria Bertani (LB) Agar

Bacteriological agar 1.5% (w/v), tryptone 1% (w/v), NaCl 1% (w/v) and yeast extract 0.5% (w/v) were dissolved in distilled water and made up to the desired volume. The mixture was heated to boiling to dissolve the agar and then sterilized by autoclaving at 121°C for 15 minutes. The agar was allowed to cool before adding the antibiotics to the desired final concentrations. The agar was then poured into sterile plates and allowed to solidify under sterile conditions. The plates were used immediately or kept at 4°C for future use.

3.3. DNA plasmid extraction from cloning host

Materials

3.3.1. GTE Solution

GTE solution was prepared by mixing 50 mM glucose, 10 mM EDTA and 25 mM Tris. The pH was adjusted to 8.0 and the solution was stored at 4°C.

3.3.2. Lysis solution

Sodium hydroxide (NaOH) 0.2 M was mixed with 1% SDS and filled to the desired volume and the solution was stored at room temperature.

3.3.3. Neutralising solution

Potassium acetate (3 M) was made using 60 ml of 5 M potassium acetate (49.07 g in 100 ml distilled water), 11.5 ml glacial acetic acid and 28.5 ml distilled water. This made up to a volume of 100 ml and the pH was 6.0. The solution was stored at room temperature.

3.3.4. TE Buffer

EDTA (1 mM) was mixed with 10 mM Tris and the pH was adjusted using HCl to 8.0. The solution was kept at 4°C.

3.3.5 Ethanol (70%)

Ethanol (70 ml) was mixed with 30 ml distilled water and stored at 4 degrees before use.

Method

E.coli DH5α bacterial cells with the cloned plasmid were cultured in LB medium with ampicillin and kanamycin, overnight in a shaking incubator at 37°C. The overnight culture was then transferred to a 1.5 ml Eppendorf tube and the cell culture was spun down for 5 minutes at 7500 rpm (7250 g) at 4°C using a small table-top centrifuge. The supernatant was carefully discarded into a conical flask and to remove the supernatant completely a paper towel was used to blot the liquid with the tube in an upside down position for a few seconds. The pellet was the resuspended into 200 µl of GTE solution and 2 µl of RNase was added and the solution was left at room temperature for 5 minutes. The suspension was transferred into a new sterile microfuge tube and 400 µl of NaOH solution was added to lyse the cells. Mixing

was done by gently flicking the tube with a finger and the tube was placed on ice for 5 minutes.

Potassium acetate solution (300 µl) was added and the solution was mixed thoroughly before placing it on ice for 5 minutes. Normally the chromosomal DNA released from the lysed bacterial cells forms a precipitate that is white in colour and this was observed before continuing. The tube was then centrifuged at 15000 g for 10 minutes at room temperature. The supernatant was transferred to a new 1.5 ml Eppendorf tube without disturbing the white precipitate using a 1 ml pipette. Then 600 µl of ice-cold isopropanol was added and the solution was stored at -20 °C for 30 minutes. The plasmid DNA precipitate was spun down at 50000 xg for 10 minutes at 4°C. The supernatant was removed completely. The precipitate containing the DNA turned white when dry and it was washed with 0.5 ml of ice-cold 70% ethanol and then the pellet was dried out in an evaporator for 2 minutes. The DNA pellet was then resuspended in 50 µL TE. The DNA was then sent for sequencing at Inqaba Biotech (South Africa) and the rest was run on agarose gel.

3.4. DNA Quantification and analysis

DNA quantification helps in the building of DNA profiles even in forensics. There is always need to use assays that are highly sensitive and give fast results which are accurate. In this research we used the qubit flourometer protocol for DNA quantification for confirmation that the DNA had been surely extracted and with good quality. Afterwards the DNA was digested and run on agarose gel to check the presence of the genes.

3.4.1. Qubit Fluorometer

This assay is a fluorescent based assay method used for detection of DNA, RNA and proteins. It incorporates mix and read protocols that make use of reagents which are also fluorescence-based. Reagents were mixed in 0.2 ml tubes that could fit into the machine. The working solution was prepared by combining 1 µl qubit reagent with 199 µl qubit buffer. The solution was vortexed for 2-3 seconds without creating bubbles. Two standards were prepared from the kit for nucleic acid assays. From the sample 5 µl were combined with 195 µl of the working solution. The tube was vortexed for 2-3 seconds to mix. The tubes were incubated for 2 minutes at room temperature. The tube was then put in the qubit machine and

the correct assay for DNA was chosen and after pressing "Go" the results were displayed on the screen.

3.4.2. Nucleotide sequencing and sequence analysis

A sample of the extracted DNA was sent to Inqaba biotech (South Africa) for sequencing. After getting the results sequence analysis and manipulations were performed using CLC Combine Workbench (CLCBIO, Denmark) and DNA Baser software with the aid of Basic Local Alignment Search tool search engine (Altschul *et al*., 1997).

3.4.3 DNA restriction digestion

Restriction digestion was done using Xhol I and Nde I for linearizing the DNA and sizing. The fast digest protocol was used in which 2 µl of 10X fast digest buffer, 1 µl of Xhol I, 1 µl of Nde I and 6 µl of nuclease free water were mixed together. The reaction mixture was incubated on a heating block at 37°C for 5 minutes.

3.4.4. Agarose Gel Electrophoresis

Preparation of Reagents

3.4.4.1. Loading dye (6X)

Distilled water (1 ml) and glycerol (1 ml) was mixed with 0.05 mg of bromophenol blue to make 6X loading dye. The loading dye helps in tracking the samples during electrophoresis. For long term storage the buffer was left frozen at -20°C.

3.4.4.2. TAE Buffer (1X)

Tris base (4.84 g) was mixed with 1.14 ml of glacial acetic acid and 2 ml of 0.5 M EDTA was added to the solution and mixed. The mixture was poured in a 1l graduated cylinder and made up to the total volume of 1l ml with distilled water.

Method

Agarose powder (1 g) was weighed out and added to a 500 ml flask. 100 ml of the 1X TAE buffer was added to the gel to make 1% agarose gel. The agarose was melted in a microwave until the solution became clear. The solution was left to cool to about 50°C. Ethidium bromide (2 µl) was added to the gel. The casting tray was assembled and sealed and the combs were placed before pouring the gel. The melted gel was poured into the casting tray and left to solidify. After solidifying the combs were carefully pulled out and the gel was

placed into the electrophoresis chamber. Enough TAE buffer was added to cover the gel. 5 µl of the DNA ladder was added to the first lane and the samples were mixed with 4 µl of the loading dye and loaded into the remaining wells. The gel was run at 90 V until the dye was about 3 quarters from reaching the end. The gel was the viewed in the GelDoc and a picture was taken.

3.5. Transformation

3.5.1. Preparation of competent cells

Bacterial cells (*E.coli* BL21 (DE3) were inoculated onto LB plates by the streak method and incubated at 37°C overnight. Preparation of the starter culture was done using a single colony from the overnight LB plates. A single colony was used to inoculate 10 ml of LB media for purification and was grown overnight in a shaking incubator. The starter culture was added to 1 L of LB media and incubated at 37 °C with shaking with the OD_{600} measured every hour and then after every 15 to 20 minutes until it got to 0.2. When the OD_{600} was between 0.35 and 0.4, the cells were immediately put on ice. The culture was then left on ice for 30 minutes and occasionally swirled. The 1 L culture was then divided into 4 equal volumes. The cells were harvested afterwards by centrifugation (3000xg, 15 mins, 4 °C). Discarding of the supernatant followed and the cell pellet was put in 100 ml of $MgCl_2$ which was kept cold. The suspensions were merged and put into a centrifuge bottle and the cells were harvested by centrifugation (2000 xg, 15 mins, 4°C). The supernatant was again discarded and the pellet was added to 200 ml ice-cold $CaCl_2$, then the suspension was left on ice for 20 minutes. Small 1.5 ml centrifuge bottles were put on ice for chilling and the cells were harvested by centrifugation (2000 xg, 15 minutes, 4 °C). A conical tube of 50 ml volume was rinsed with ddH_2O and placed on ice. The supernatant was thrown away and the cell pellet put in 50 ml ice cold 85 mM of $CaCl_2$ and 15% glycerol. The suspension was then transferred into a conical tube. The cells were harvested by centrifugation (1000 xg, 15 mins, 4°C). The supernatant was discarded and the pellet resuspended in 2 ml ice cold 85 Mm $CaCl_2$ and 15% glycerol. The OD_{600} of the suspended cells was measured and it was between 200 and 250. The cells were then transferred (50 µl) into a sterile 1.5ml microfuge tube and frozen with liquid nitrogen. The cells were finally frozen at -80°C.

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3.5.2 Transformation of the competent cells.

Competent cells kept in the -80°C freezer were thawed on ice for 20 minutes and agar plates containing ampicillin or kanamycin were taken out of the 4 °C fridge so that they could warm up at room temperature. Plasmid DNA (1 µl) was mixed with 50 µl of competent cells in the tube. Mixing was done gently by flicking the tube with a finger. The mixture was left on ice for 30 minutes. The tube was then heat shocked and placed back on ice for 2 minutes. The SOC media (250-500 µl) without the antibiotic was mixed with the cells and incubated at 37 °C with shaking for 45 minutes. The transformation mixture was added to LB agar plates containing ampicillin or kanamycin and incubated at 37 °C overnight. Single colonies were present on the plates in the morning indicating successful transformation. As a control BL21 cells without the plasmid were also grown on the plates and absence of any growth showed that the antibiotics were working.

3.6. Protein Expression

Materials

3.6.1. Isopropyl β-ᴅ-1 thiogalactopyranoside

The IPTG powder (0.238 g) was weighed out and dissolved in 10 ml of distilled water. Sterilization was done by filtering the mixture through a 0.22 µm filter. The solution was then aliquoted into 1 ml Eppendorf tubes and stored at -20°C for future use.

3.6.2. Lysis buffer

Tris (25 mM) was mixed with 2 mM of EDTA and the pH was adjusted with HCl to 7.6. The buffer was mixed with bacterial cell at a volume of 1:20 before freezing.

Method

The cultures of *E.coli* BL21 (5 ml) grown overnight harbouring the plasmids with the esterase genes were used to inoculate 50 ml of LB broth/ampicillin (100 µg/ml). The cultures were incubated at 37°C until the optical density was between 0.6 and 0.8. Induction was initiated by addition of IPTG which had a concentration of 1 mM and the cultures were further incubated at 25 °C for 5 h, followed by centrifugation (8000 xg, 10 min, 4 °C). Harvested cells were resuspended in 10 ml lysis buffer for protein extraction and left for 10 minutes at room temperature. The cells were then frozen at -80 overnight and then thawed in the morning to burst the cells. The cells were sonicated for 5 minutes at 30 seconds intervals

while on ice and then centrifuged for 20 minutes at 15000xg after which the pellet was discarded and the supernatant was used as the intracellular protein source.

3.7. Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE)

The tricine-SDS-PAGE protocol was used by Schägger (2006) because it allows higher resolution in smaller proteins. It is commonly used to separate proteins that are between 1 and 100 kDa. Acrylamide concentrations are much lower compared to other electrophoretic systems. Viewing of the proteins was enabled by staining with Coomassie blue G-250 (Dennison, 2003). A picture was then taken using the geldoc system for the record of results.

Reagents preparation

3.7.1. Ammonium Persulphate

Ammonium persulphate 10% (w/v) was made up by dissolving 0.2 g in 2 ml of distilled water and stored at 4 °C.

3.7.2. Monomer solution [30% (w/v) acrylamide, 2.7% (w/v) bis-acrylamide]

Acrylamide (73 g) was weighed out and put in a graduated cylinder and bis-acrylamide (2 g) was also weighed out and added to the same cylinder. The two were dissolved and the volume made up to 250 ml with distilled water. The solution was filtered and stored in a dark bottle at 4 °C.

3.7.3. Running Gel buffer (4X) [1.5 M Tris HCl, pH 8.8]

Tris (45.37 g) was dissolved in 200 ml of distilled water and the pH was adjusted to 8.8 with HC and then the volume was made up to 250 ml with distilled water. The solution was filtered and stored at 4 °C.

3.7.4. Stacking gel buffer (4X) [500 mM Tris HCl, pH 6.8]

Tris (3 g) was dissolved in 40 ml distilled water and the pH adjusted to 6.8 with HCl and the volume made up to 50 ml with distilled water. The solution was filtered and stored at 4°C.

3.7.5. Sodium dodecyl sulphate (SDS)

SDS powder (10 g) was weighed out to make a 10 % (w/v) solution and dissolved in 100 ml of distilled water. The mixture was gently heated.

3.7.6 Anode buffer [0.2 M Tris-HCl, pH 8.9]

Tris (24.2 g) was dissolved in 950 ml of distilled water and the pH was adjusted to 8.9 with HCl. The volume was then made up to 1000 ml with distilled water.

3.7.7. Cathode buffer [0.1 M Tris-HCl, 0.1 M tricine, 0.1% (m/v) SDS pH 8.25]

Tricine (17.9 g) was mixed with 12.1 g Tris and 800 ml distilled water and the pH was adjusted to 8.25 with HCl. SDS [10 ml of 10% (m/v)] was added and the volume was made up to 1000 ml.

3.7.8 Treatment buffer [125 mM Tris-HCl, 4% (m/v) SDS, 20% glycerol, 10% (v/v) 2-mercaptoethanol, 0.01% bromophenol blue, pH 6.8]

Glycerol (2 ml) was mixed with stacking gel buffer (2.5 ml) and 4 ml of 10% SDS together with 1 ml of 2-mercaptoethanol and 0.01% (m/v) of bromophenol blue and distilled water was added to a final volume of 10 ml.

3.7.9. Coomassie Blue Stain [0.125% (m/v) Coomassie brilliant blue G-250, 50% (v/v) methanol, 10% (v/v) acetic acid]

Coomassie brilliant blue G-250 powder (1.25 g) was mixed with 500 ml methanol and 100 ml of acetic acid and volume was made up to 1000 ml with distilled water.

3.7.10. Destain I

Methanol (500 ml) was mixed with acetic acid 100 ml and the volume was made up to 1000 ml with distilled water.

3.7.11. Destain II

Methanol 5% (v/v/) was mixed with acetic acid 7% (v/v) and the volume was made up with distilled water.

Method

Firstly the electrophoresis unit was assembled and the running gel solution was prepared. Running gel solution consisted of distilled water 5.05 ml, monomer solution 6.67 ml, gel buffer (4X) 4 ml, APS 0.016 ml and TEMED 0.240 ml. Carefully the solution was poured in between the glass plates and immediately overlaid with distilled water. The gel was left to set for 30 minutes and then the water was removed with a syringe. The stacking gel solution was prepared by mixing distilled water 3 ml, monomer solution 0.67 ml, gel buffer (4X) 1.25 ml, APS 0.010 ml and TEMED 0.050 ml. The solution was carefully poured on top of the running gel in-between the plates. The gel was left to solidify for 20 minutes. Equal volumes of samples were mixed with equal volumes of the treatment buffer and the samples were put on a heating block at 95°C for 3 minutes and then they were loaded. Electrophoresis was run at a voltage of 120 V until the gel was 1 cm from the bottom. The electrophoresis equipment was completely disconnected from power supply and the gel was carefully removed from the plates and stained overnight in Coomassie blue stain. Subsequently the gel was destained in destain I until the gel background was clear and then into destain II and the gel was then viewed on the geldoc system and a picture was captured of the results.

3.8. Enzyme screening for activity

The crude enzyme mixtures were first checked for esterase activity using two different substrates: naphthol AS-D chloroacetate and α-naphthylbutyrate. The reaction mixture would change colour from blue to purple if the enzymes were active.

3.8.1. Esterase activity assays

Esterase activity determinations were performed in triplicate. Enzyme activity measurement was done by considering that 1 unit is the amount of enzyme releasing 1 µmol of *p*-nitrophenol per minute from *p*-nitrophenyl acetate. Specific activity was given in terms of the number of units per milligram of protein. The reaction (1 ml) was carried out in Sodium phosphate buffer (50 mM, pH 7.0) that contained 1 mM *p*-nitrophenyl acetate (dissolved in isopropanol and kept on ice). The reactions were started by the addition of 50 µl of the crude enzyme solution. Incubation was done at 40°C for 5 minutes and the addition of 200 µl HCL (36% w/v) stopped the reaction. The identical reaction mixture excluding the enzyme was included as a control to correct for the autohydrolysis of the substrate.

3.8.2. Biochemical characterisation

All biochemical characteristics of the 14 enzymes were determined using *p*-nitrophenyl acetate (1 mM) as a substrate. The temperature optima of the enzymes were determined between 30°C and 80°C. The standard reaction mixture was incubated at the desired temperatures and the reactions started by adding 50 µl enzyme volumes.

3.8.2.1 Effects of temperature

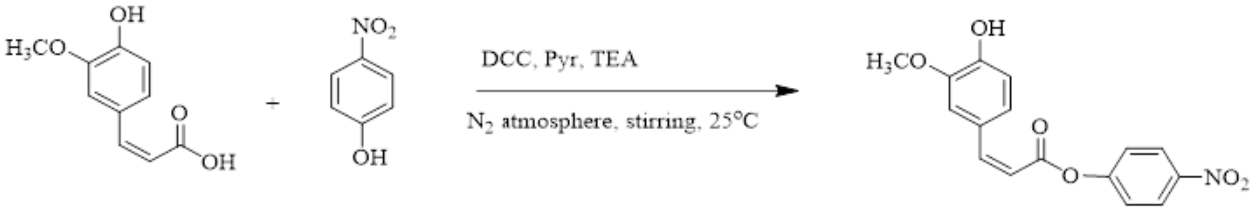
The thermostability profiles of the enzymes were determined by incubating the enzymes in Sodium phosphate buffer (100 mM, pH7.0) at temperatures ranging from 10°C to 80°C. The residual activities were determined at timed intervals by measuring the release of *p*-nitrophenol.

3.8.2.1 Effects of pH

The effect of pH was measured between the ranges of 3 to 11 using a 50 mM Sodium phosphate buffer. (Britton and Robinson., 1931).

3.8.3 Chemical Synthesis of 4-Nitrophenyl Ferulate

For measurement of esterase activity another spectrophotometric assay method was used for easy, quick and routine assays using 4-nitrophenyl ferulate (4NPF). The results were compared to those for the cleavage of *p*-nitrophenyl acetate for certainty that the enzymes were active. Because 4-nitrophenyl ferulate is not commercially available we synthesised the compound using chemical procedures described by Hedge *et al* (2009). The starting compounds were 4-nitrophenol and ferulic acid. 4NPF was coupled with ferulic acid using the coupling agent dicyclohexylcarbodiimide (DCC). The reaction is shown below in Scheme 3.1.



Scheme 3.1: Chemical synthesis of 4-nitrophenyl ferulate.

Ferulic acid (1 g (5.15 mmol, melting point 168-170°C)) was dissolved in dioxane (10 ml) and 4-nitrophenol 0.716 g (5.15 mmol, melting point 112-114°C). The reaction was made

basic by adding pyridine 1 ml and triethylamine 1 ml. The reaction was mixed by stirring for 10 minutes. Solid DCC was added 1.18 g (5.7 mmol) in portions of 200 mg at 3 minutes intervals under a nitrogen atmosphere at 25°C. The reaction was then monitored by thin layer chromatography and the precipitate of dicyclohexyl urea started appearing after about 15 minutes. The complete disappearance of the starting materials signalled the completion of the reaction. The developing agent was methanol in chloroform (10%). The reaction took about 9 hours after which DCU was removed by filtration and filtrate evaporated. Column chromatography using silica gel (100-200 mesh size) with 10% methanol in chloroform as eluent was used to purify the 4NPF. Fractions with an R_f value of 0.72 were pooled, evaporated and quantified. All the chemicals used were analytical grade and obtained from Sigma-Aldrich, Germany. The final product was analysed by HPLC. 4NPF was further characterised by nuclear magnetic resonance (NMR).

3.8.4 Substrate specificities

The substrate specificity preferences of the esterases were determined using *p*-nitrophenyl esters following the description by Rashamuse *et al*., (2009). Specified *p*-nitrophenyl esters with different chain lengths were used to determine the substrate specificity in 1mM concentrations: *p*-nitrophenyl acetate (C2), *p*-nitrophenyl butyrate (C4), *p*-nitrophenyl valerate (C5), *p*-nitrophenyl octanoate (C8), *p*-nitrophenyl deodecanoate (C12) and *p*-nitrophenyl palmitate (C16). Experimental initial velocity data vs. substrate concentration (with coefficient variation of <5%) was used for calculations in a Michaelis-Menten equation by using hyperbola analysis.

3.9. Biocatalysis

There are a few drugs that can be mentioned, such as naproxen, where esterases have been used to obtain enantiopure products by means of enzymatic resolution. Most of the enzyme catalysed reactions are not good enough for commercialisation, leading to a great need to look into these types or reactions and biocatalysis in general in order to improve enantioselectivity. In an attempt to do this 3 different compounds were studied and their racemic or diastereomeric mixtures were exposed to esterases and lipases to check for selective hydrolysis. The different reactions were set up under various conditions for maximum selectivity.

3.9.1 Enzymatic resolution of racemic mixture from the Morita-Baylis-Hillman reaction

i) Hydrolysis of Methyl 3-acetoxy-2-methylene-5-phenylpentanoate (Compound 1)

All the enzymes were kept on ice to avoid denaturing. The reaction buffer which was sodium phosphate buffer 100 mM was prepared by adding 1.56 g of sodium salt to 100 ml of distilled water. The pH was adjusted to 7.0 using NaOH. 800 µl of the buffer was added to a sterile Eppendorf tube followed by 100 µl of enzyme and the reaction was started by adding 100 µl of the substrate. The reactions were left at 40°C for 48 hours. The reaction mixture was then worked up and analysed by HPLC. For the analytical HPLC a Dionex Ultimate 3000 instrument was used, employing a Luna C18 (2) 100A, 250 x 4.6mm column, at a flow rate of 1.0 ml/min with detection at 217 nm using Acetonitrile: water (90:10) as a solvent system.

ii) Hydrolysis of 2-cyano-5-phenylpent-1en-3-yl acetate (Compound 2)

The buffer chosen to conduct hydrolysis experiments was potassium phosphate 50 mM at pH 7.2. 52 mg of the cyano derivative compound 2 was added to 750 µl of acetone. 50 µl of this was then taken and mixed with 850 µl of the potassium phosphate buffer and 100 µl of enzyme. The reactions were first conducted at 20 °C and the temperature was then changed to 40 °C to improve hydrolysis. Analytical HPLC was done using a CHIRALCEL OJ column type, lot number 168-059-40615 from DAIICEL chemical industries limited, at a flow rate of 1.5 ml/min using a Methane:Hexane:IPA (5:70:25) solvent system.

3.9.2 Enzymatic resolution of diastereomers from the Passerini reaction

i) Hydrolysis of (2*S*,3*S*)-3-((*tert*-butoxycarbonyl)amino)-1-(butylamino)-1-oxo-4-phenylbutan-2-yl acetate

The reactions were set at 40 °C after mixing 800 µl of sodium phosphate buffer at pH 7.5 with 100 µl of enzyme and 100 µl (60 mg/ml) of substrate. The reaction was shaken for 48 hours and was then checked using HPLC analysis. For all analytical HPLC a Dionex Ultimate 3000 instrument was used, employing a Luna C18 (150 x 4 mm) reverse phase column gradient elution, at a flow rate of 1ml/min with detection at 217 nm.

CHAPTER 4: RESULTS

4.1. General properties of the esterase genes

The 14 esterase genes cloned using *E.coli* DH5-α had been previously isolated from various sources including metagenomic libraries and termites hindgut. The Table 4.1 shows the general properties of the 14 characterised genes. The table shows the sizes of the genes ranging from 783bp to 1545bp with protein sizes between 28.8kDa to 55.1kDa. The plasmids carrying the esterase genes were received from CSIR Biosciences together with the information that all the genes except for Est11, had been directionally ligated into pET20b(+) in order to allow for the expression of the genes under a strong T7 promoter and also a His-tag sequence had been inserted into the plasmid for previous purification. Est11 was ligated into pET28a (+) plasmid also selective using a T7 promoter. All the proteins after expression were produced in the soluble cytoplasmic fraction of the *E.coli* BL21 cells. The fractions with the proteins were not purified and were used in the form of crude extracts as the research was aiming at studying reactions that can be later increased to industrial scale and elimination of a purification step will be crucial and cost effective if the enzymes can just work as their crude extracts. Purification of enzymes at large scale is expensive and usually leads to unfolding of proteins and the refolding will be both time consuming and expensive. Sequencing of the genes showed that they were within frame. Table 4.1 also shows the various sources of the esterases which were mainly from termite hindguts and the Chloorkop landfill in the East Rand of Johannesburg, South Africa.

Table 4.1: General properties of the 14 esterase genes.

Original Name	**Identity**	**Type of Esterase**	**Source**	**Gene size (bp)**	**Molecular Weight of Protein (kDa)**	**Reference**
P20 Fae1	EST 1	Feruloyl esterase	Termite hindgut	825	31.4	Rashamuse *et al*., 2014
P20 Fae2	EST 2	Feruloyl esterase	Termite hindgut	783	29.8	Rashamuse *et al*., 2014
P20 Fae3	EST 3	Feruloyl esterase	Termite hindgut	792	28.9	Rashamuse *et al*., 2014

P20 Fae4	EST 4	Feruloyl esterase	Termite hindgut	801	31.1	Rashamuse *et al*., 2014
P20 Fae5	EST 5	Feruloyl esterase	Termite hindgut	804	30.1	Rashamuse *et al*., 2014
P20 Fae7	EST 6	Feruloyl esterase	Termite hindgut	786	29.6	Rashamuse *et al*.,2014
P28 DeAxyl	EST 7	Carboxylesterase	Termite hindgut	783	28.8	Rashamuse *et al*., 2012
P20 Est6	EST 8	Carboxylesterase	Termite Hindgut	1257	46.7	Rashamuse *et al*., 2012
P20 EstC	EST 9	Family VII esterase	Leachate metagenomic library	1281	46.3	Rashamuse *et al*., 2009
P20 Fbff	EST 10	Carboxylesterase	Chloorkop landfill	1272	45.0	Rashamuse *et al*., 2013
P28 AxeB	EST 11	Accessory Acetylxylan esterase	Termite Hindgut	1521	54.1	This study
PLysate P20 43B	EST 12	Feruloyl esterase	Leachate metagenomic library	1350	55	Rashamuse *et al*., 2011
Plysate P20 C1444	EST 13	Carboxylesterase	Termites hindgut	1545	49.1	Rashamuse *et al*., 2012
P20 cell 13	EST 14	Carboxylesterase	Termites hindgut	1377	54.1	Rashamuse *et al*., 2012

4.2. Protein expression

Introduction of the plasmids into the *E. coli* cells was done chemically using calcium chloride to transform competent cells. This allowed the cells to take up the plasmids carrying the desired genes for expression. *E. coli* BL21 cells were induced with isopropyl β-D-1 thiogalactopyranoside (IPTG) for expression. Expression of the proteins was confirmed by

running the crude extract samples on Sodium dodecyl sulphate Poly acrylamide gel electrophoresis (SDS-Page). The SDS-Page analysis showed distinct bands of the proteins (Figure 4.1 and Figure 4.2). The aim was at introducing the use of crude extracts of protein in the industry as long as the overexpressed protein is active to cut costs on purification at large scale plants. SDS-Page denatures the proteins but it allows confirmation of the protein sizes as protein molecules of different sizes travel through the gel at different speeds, allowing separation of the proteins. The SDS-Page pictures also show other proteins expressed by the *E. coli* cells but they do not influence the activity of the esterases. The expressed proteins showed respective bands on SDS-Page which were in agreement with the theoretical molecular masses sited in the references in Table 4.1.

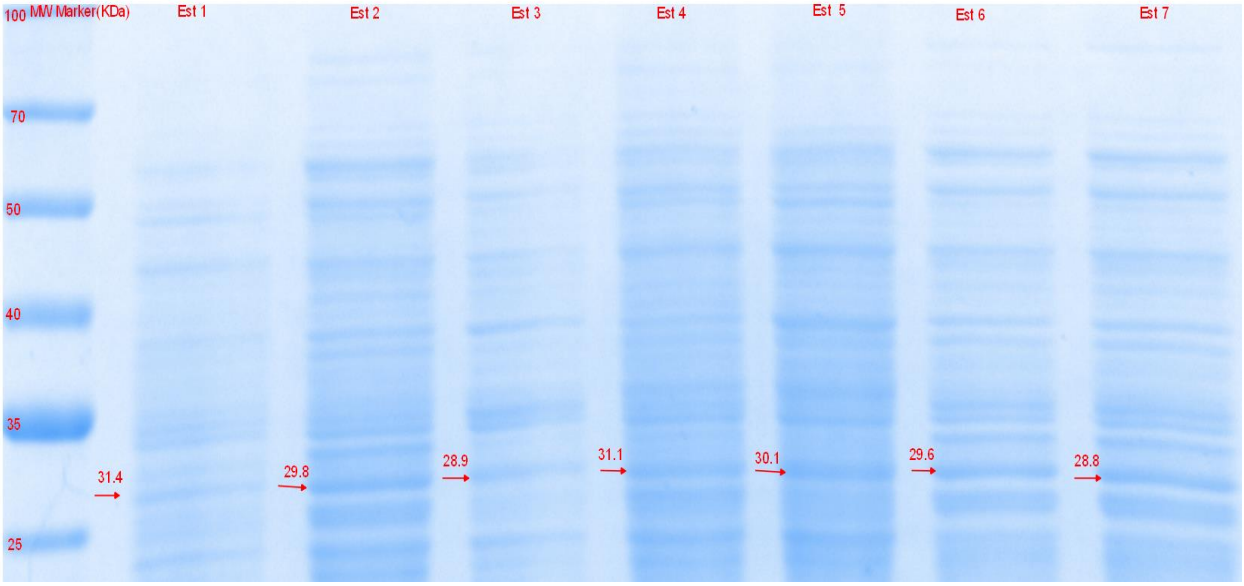


Figure 4.1: SDS-Page results of the expressed proteins Est1-Est7

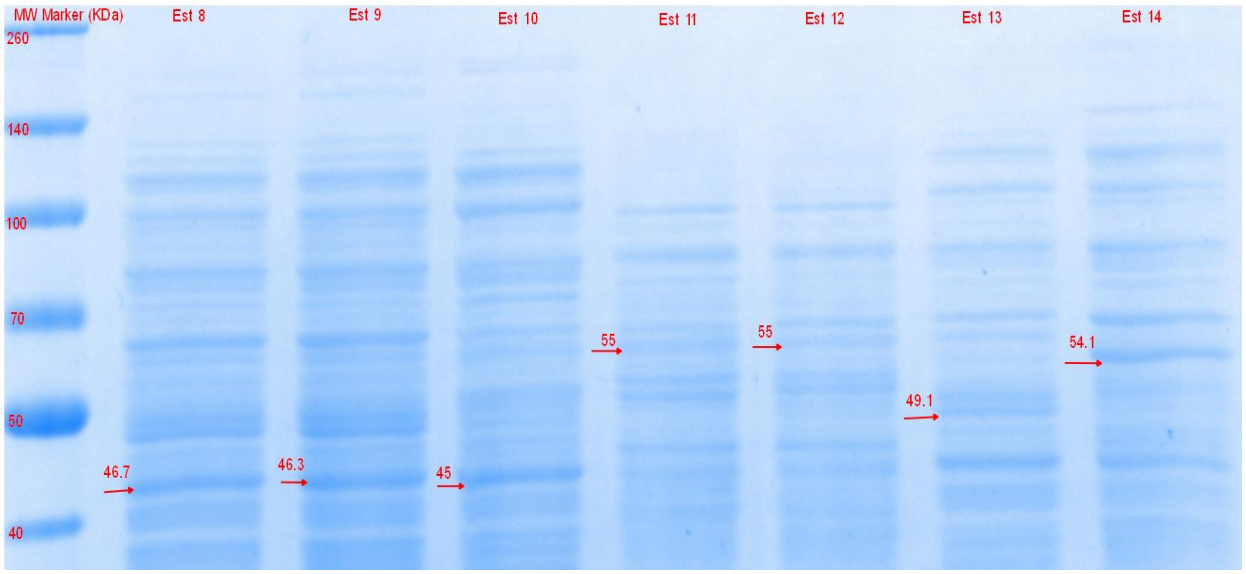


Figure 4.2: SDS-Page results for the expressed protein Est8-Est14

4.3. Protein activities

The quick test to confirm if the expressed enzymes were working was done using 4-nitrophenyl acetate. When 4-nitrophenyl acetate was put in a reaction mixture with the enzyme and buffer the mixture turned yellow within 2 minutes. The enzymes changed colour immediately showing that the esterases were active and in *E. coli* cells these are the only enzymes that can hydrolyse the substrate. This was confirmed after running a parallel reaction as a control with non-induced cells and the reaction mixture remained colourless.

4.3.1 Synthesis of 4-Nitrophenyl Ferulate

A spectrophotometric assay using 4-nitrophenyl ferulate (4NPF) was used to measure the activity of all the different varieties of esterases because it is easy and quick unlike using high performance liquid chromatography (HPLC). The 4NPF substrate is not commercially available therefore, it was synthesised by a chemical procedure (Hegde *et al*., 2009). Scheme 3.1 shows the single step synthesis of 4NPF using the starting materials ferulic acid and 4-nitrophenol (4NP). For confirmation that the substrate had been synthesised ^{1}H NMR analysis was done and the spectral data was compared to previous work done by Hegde *et al*., (2009). Figure 4.3 shows the spectral data of the 4-nitrophenyl ferulate synthesised.

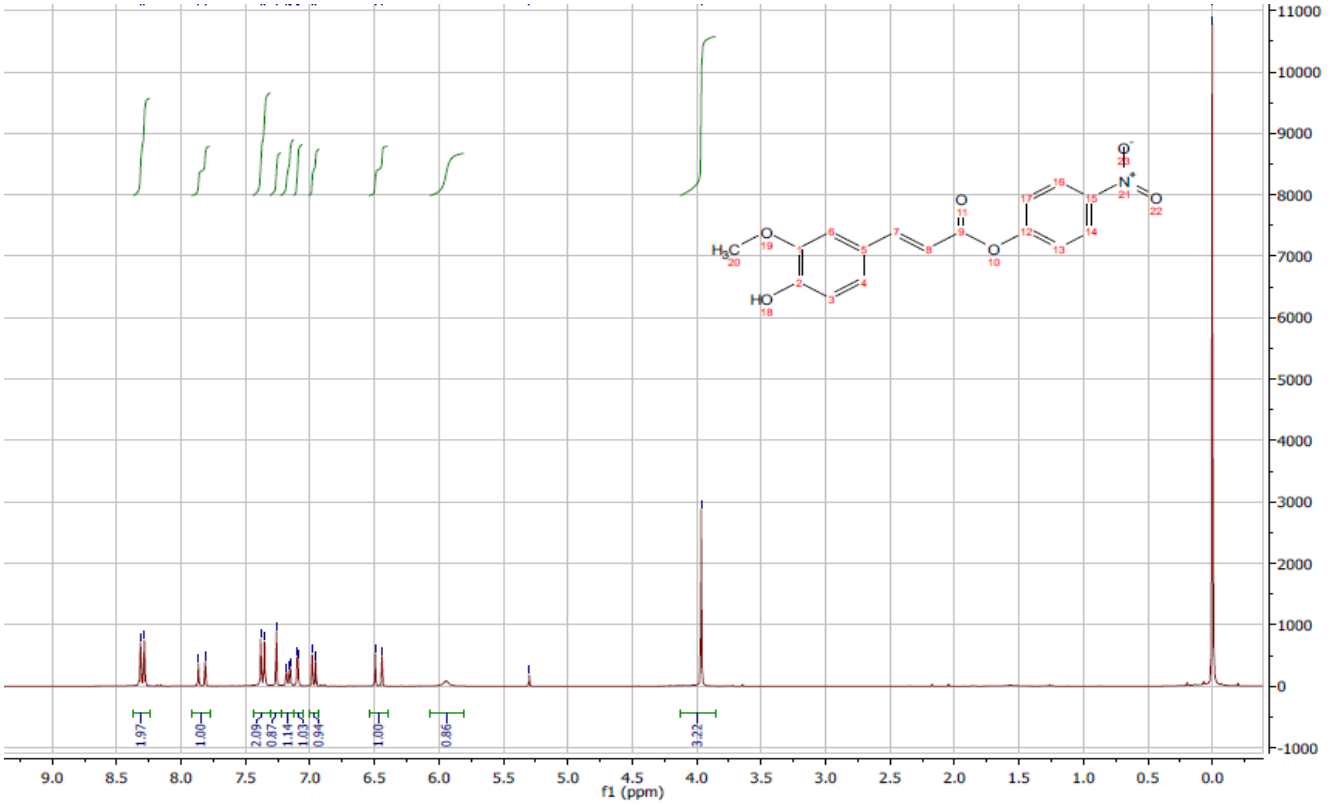


Figure 4.3 Spectral data confirming the synthesis of 4-nitrophenyl ferulate.

4.3.2. Biochemical Characterisation

4.3.2.1 pH profiles

The crude enzyme extracts of the 14 varieties of esterases showed a wide range of pH optima from 6.0 to 9.0 (Figure 4.4a to Figure 4.4m). Six of the esterases had an optimum pH of 8.0. There was low enzyme activity under acidic conditions by the enzymes. Est10, Est11 and Est13 showed highest activity at pH 9.0 (Figures 4.4j, 4.4k and 4.4m) which was not expected as the reaction will be more alkaline. Most enzymes show activity deterioration at such high pH values.

Table 4.2: Optimum pH values of the crude enzyme extracts.

Enzyme	Est1	Est2	Est3	Est4	Est5	Est6	Est7	Est8	Est9	Est10	Est11	Est12	Est13	Est14
pH	6.0	8.0	7.0	8.0	8.0	8.0	8.0	6.0	7.0	9.0	9.0	6.0	9.0	8.0

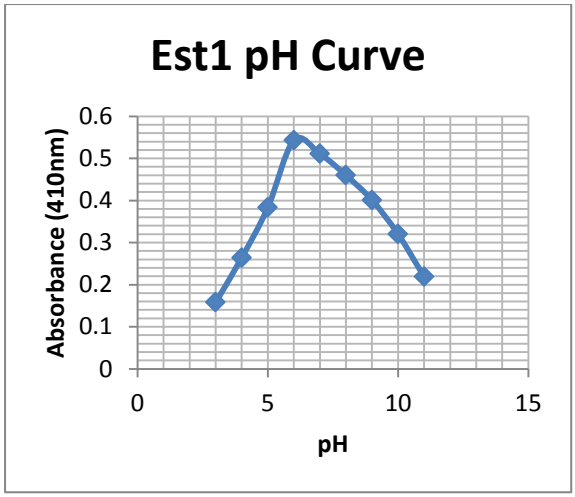


Figure 4.4a

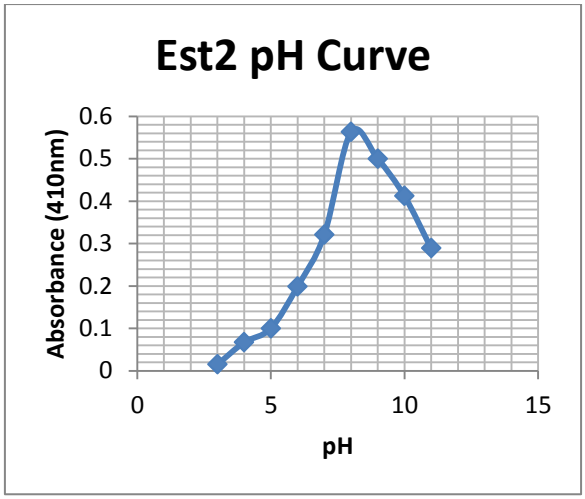


Figure 4.4b

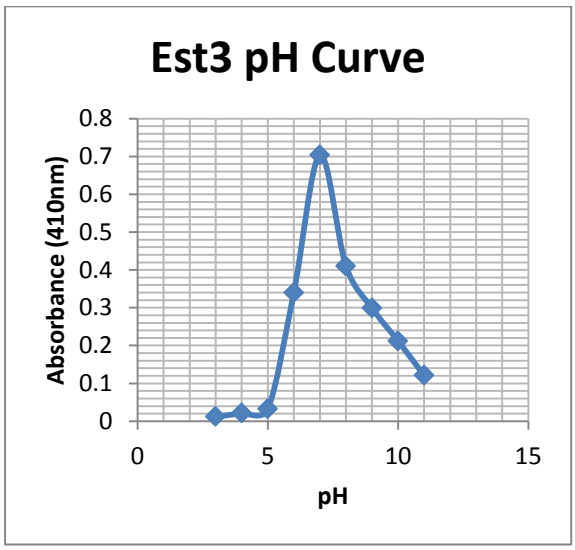


Figure 4.4c

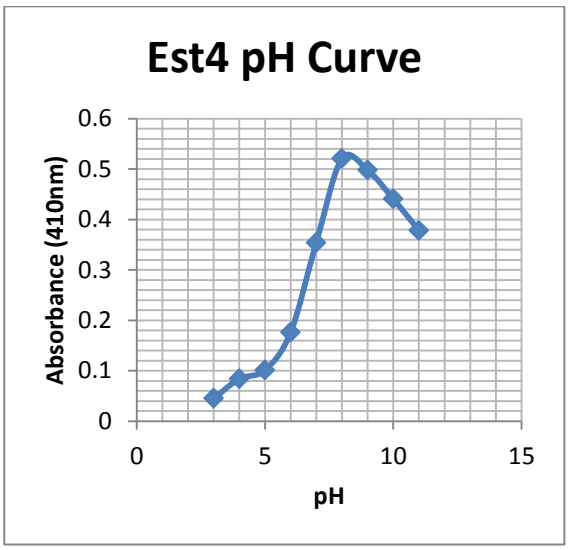


Figure 4.4d

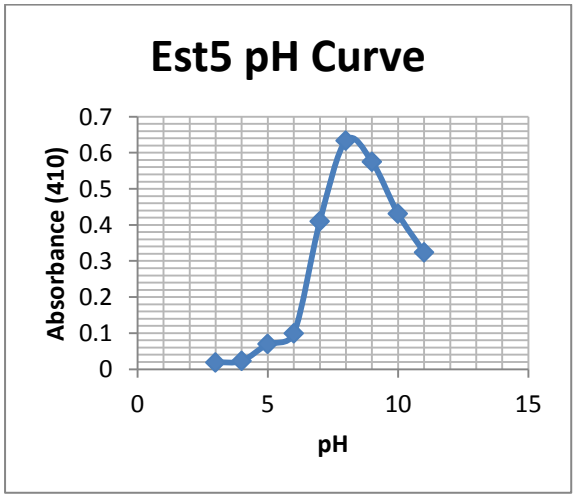


Figure 4.4e

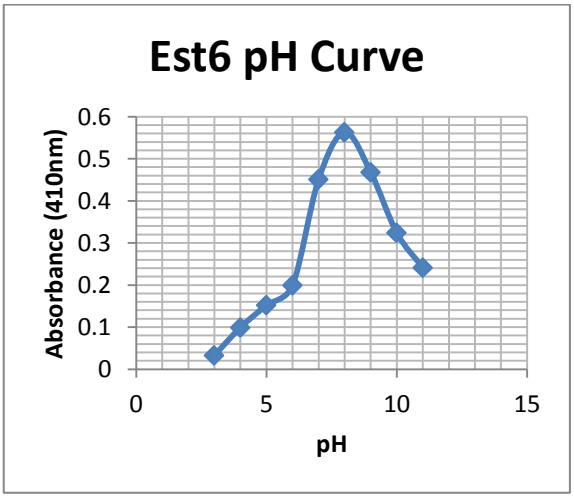


Figure 4.4f

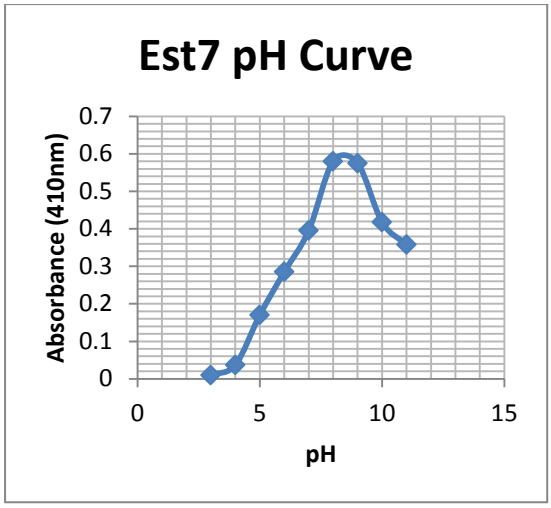


Figure 4.4g

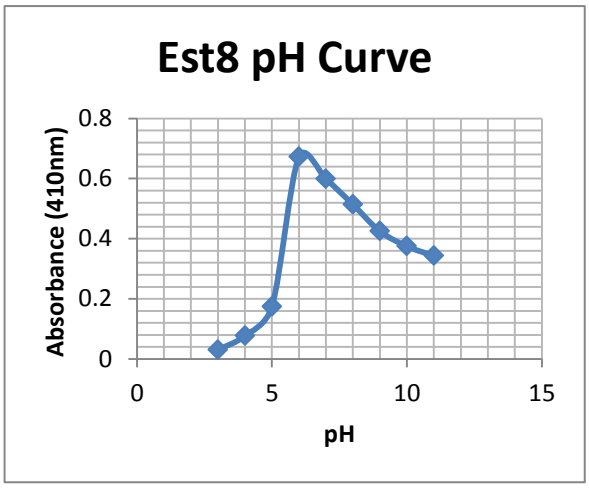


Figure 4.4h

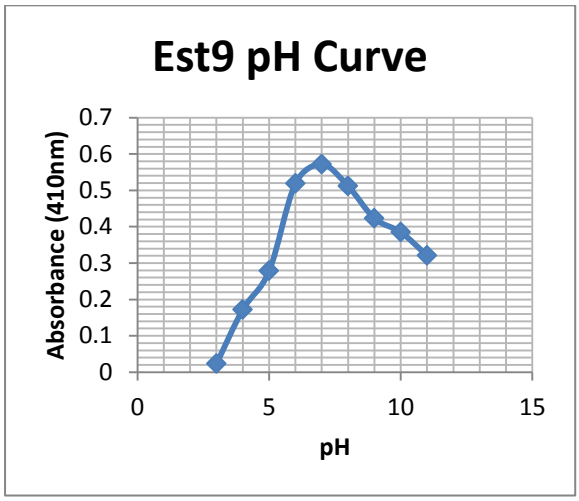


Figure 4.4i

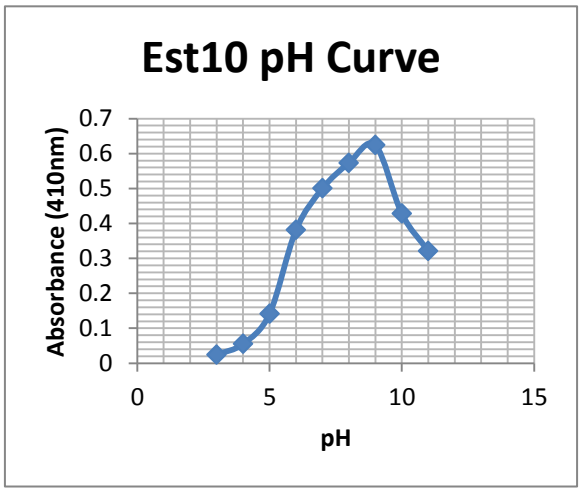


Figure 4.4j

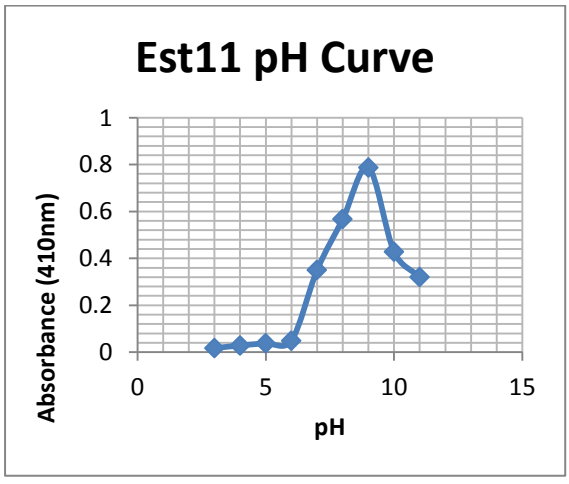


Figure 4.4k

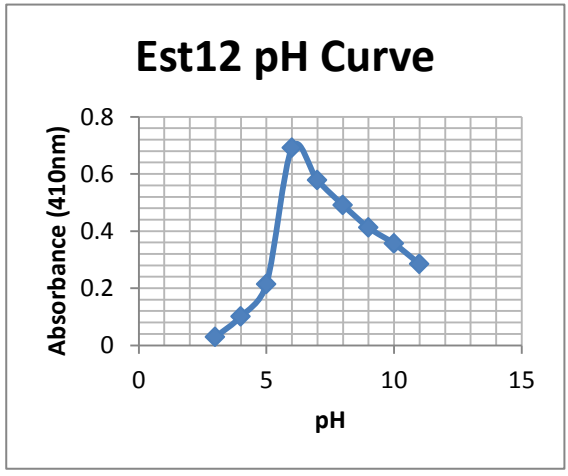
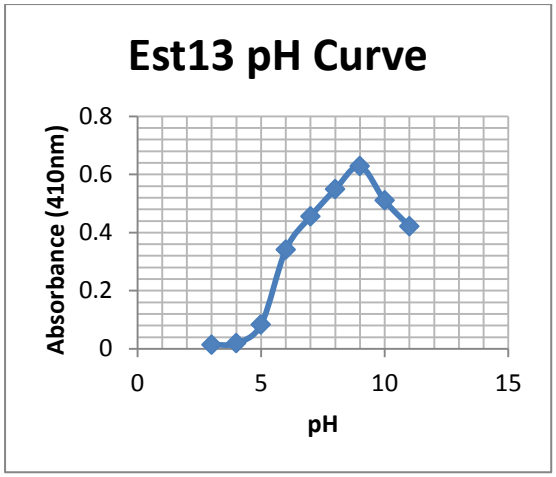


Figure 4.4l



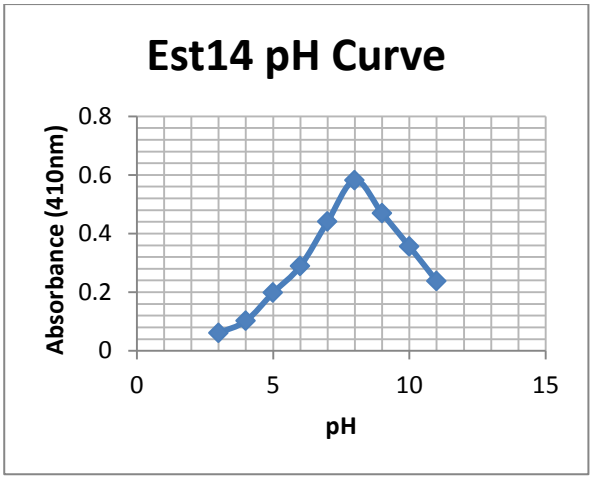
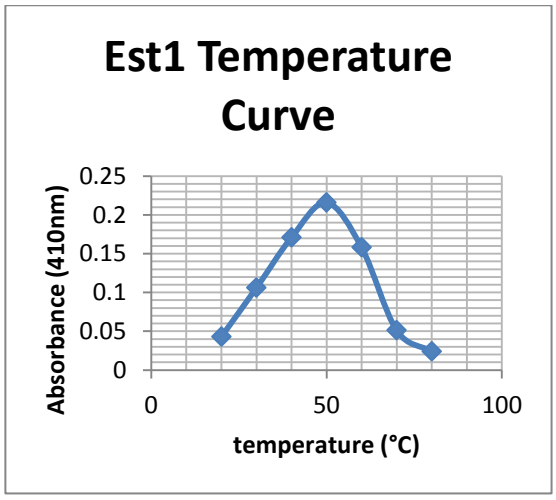


Figure 4.4m **Figure 4.4n**

4.3.2.2 Temperature profiles

The crude esterase extracts showed temperature optima of 30°C for Est4 (Figure 4.5d) and Est6 (Figure 4.5f), 35°C for Est9 (Figure 4.5i) and Est13 (Figure 4.5m), 35.5°C for Est 2 (Figure 4.5b), 38°C for Est7 (Figure 4.5g), Est8 (Figure 4.5h) and Est11 (Figure 4.5k), 40°C for Est3 (Figure 4.5c), Est5 (Figure 4.5e), Est10 (Figure 4.5j), Est12 (Figure 4.5l) and Est14 (Figure 4.5n) and 50°C for Est1 (Figure 4.5a). There was significant increase in enzyme activity at lower temperatures between 20°C and 50°C but a sharp decrease in enzyme activity was observed at higher temperatures. Enzymes that show stability over a wide range of temperature changes are of major importance in the industry as there are different and usually high temperature in the manufacture of many products in both the food and pharmaceutical industries.



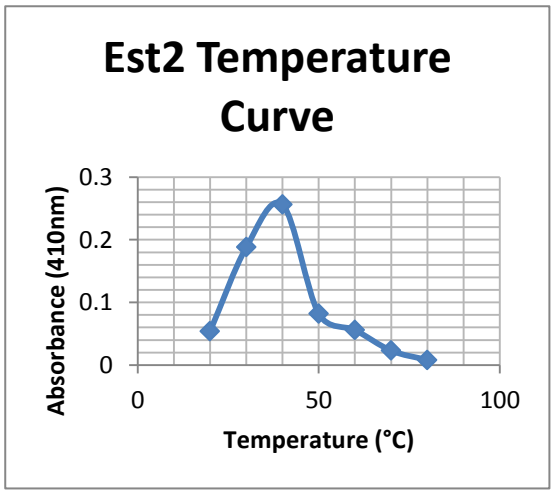


Figure 4.5a **Figure 4.5b**

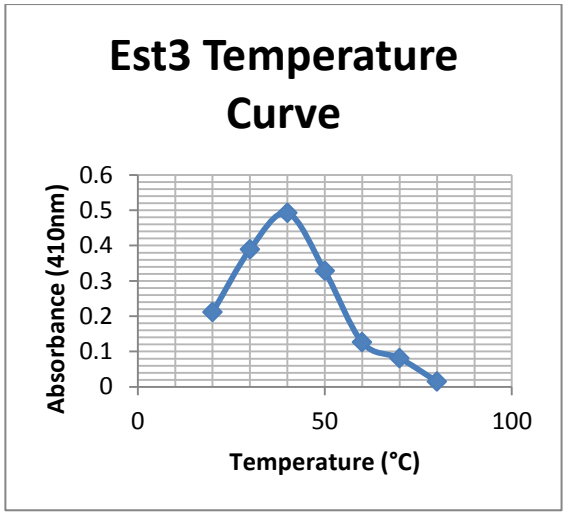


Figure 4.4c

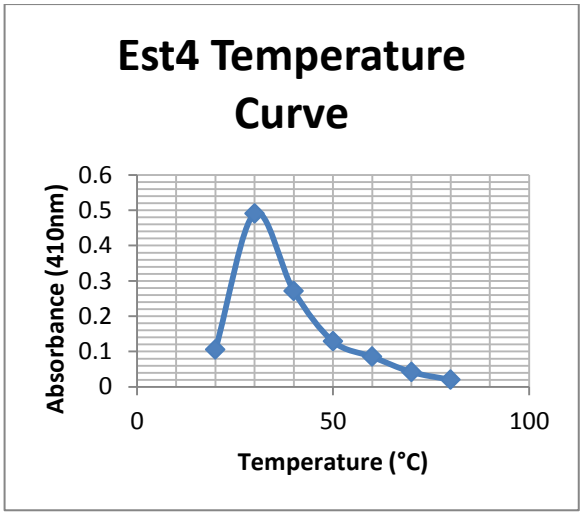


Figure4.4d

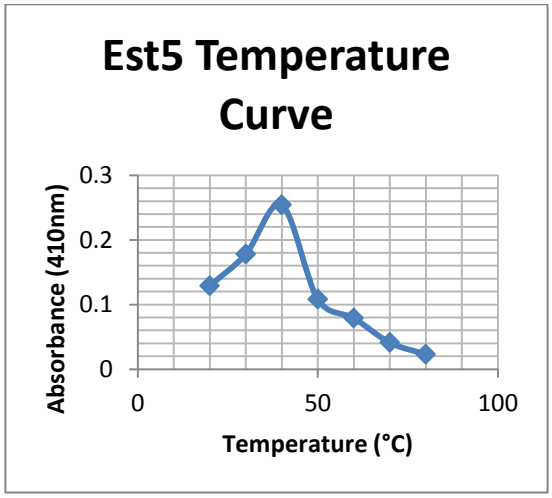


Figure 4.5e

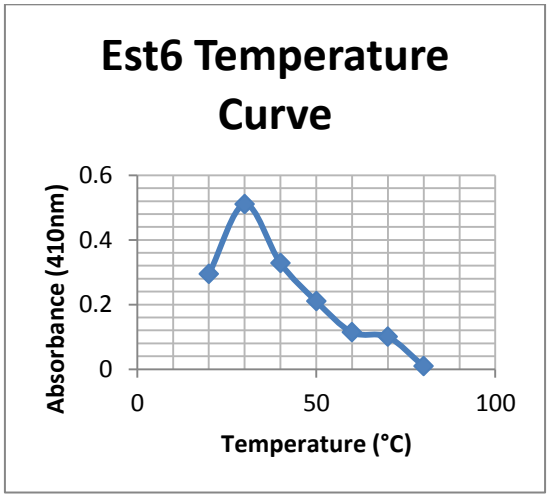


Figure 4.5f

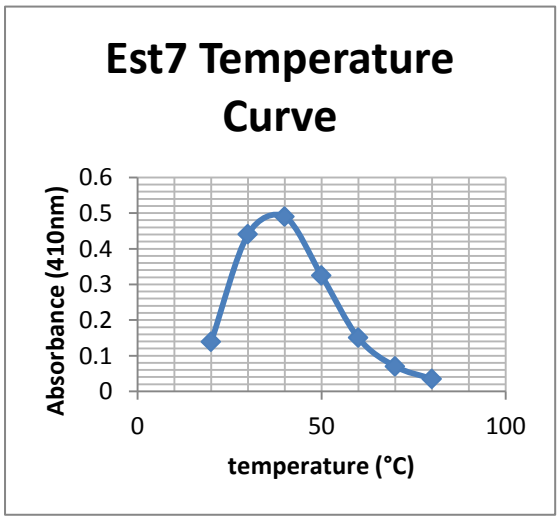


Figure 4.5g

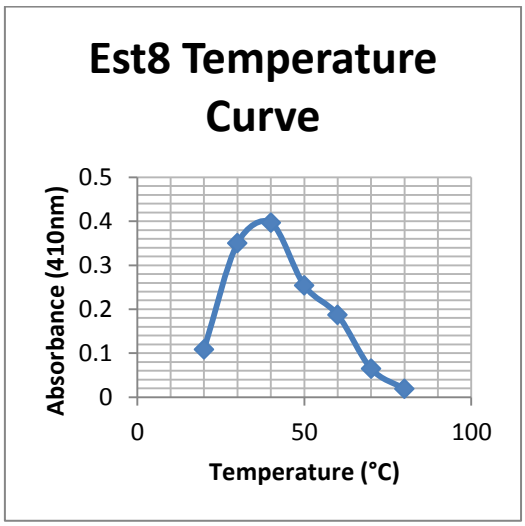


Figure 4.5h

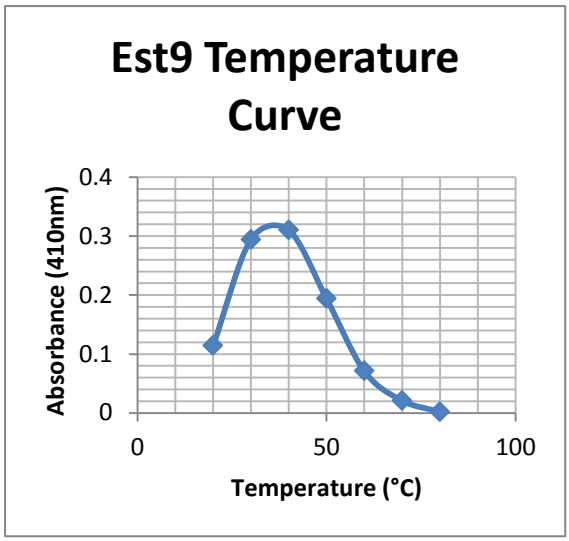


Figure 4.5i

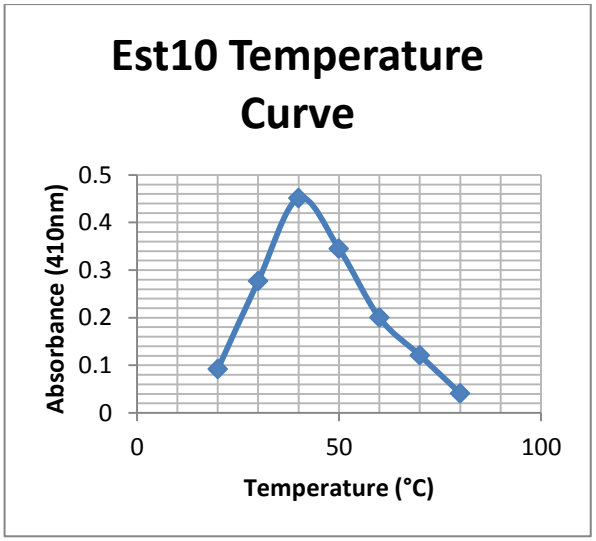


Figure 4.5j

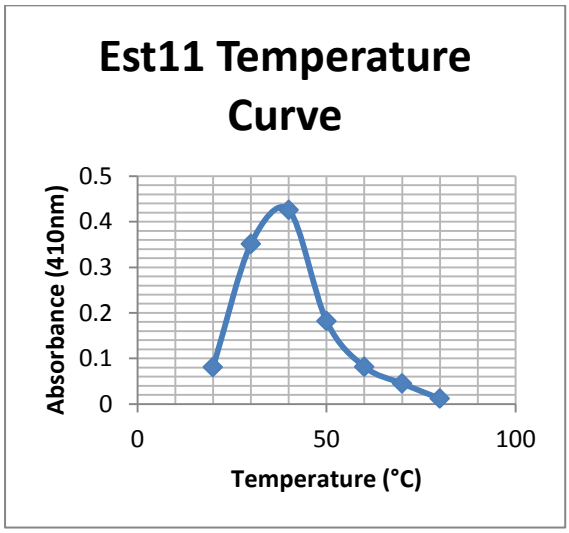


Figure 4.5k

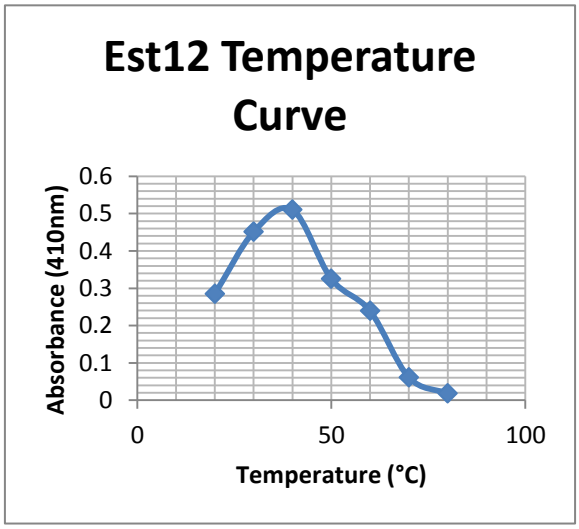


Figure4.5l

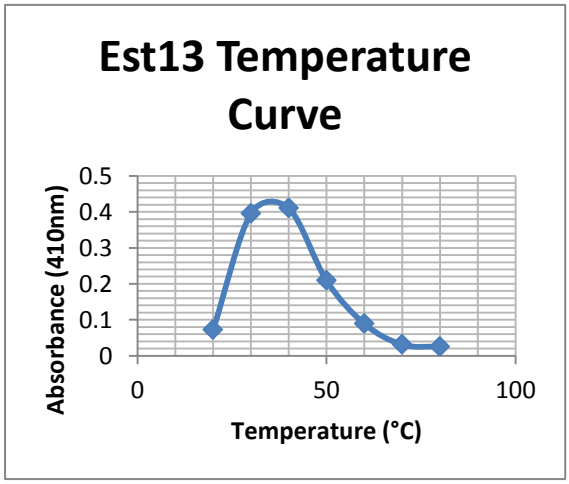


Figure 4.5m

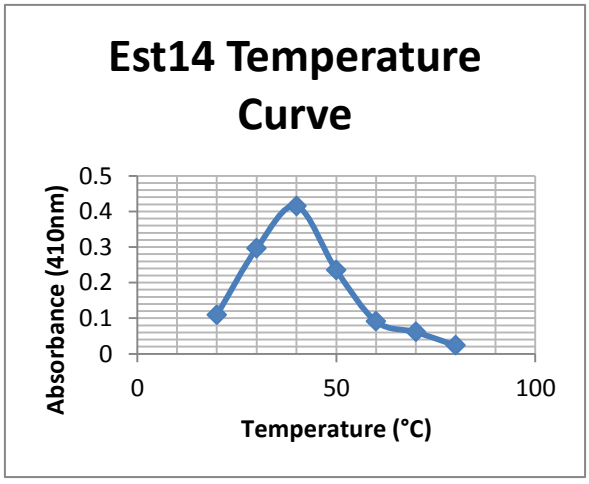


Figure 4.5n

4.4. Enzyme Kinetics

All enzyme activity assays were performed in triplicate using standard conditions and were incubated for 10 mins at 40°C. The initial rates of hydrolysis were determined for the different esterase substrates and subsequently the activities of each enzyme against the different substrates were calculated. Table 4.3 shows the calculated activities of the various enzymes on different substrates and the results showed that as the acyl chain length of the substrate increased the activity of the esterases decreased up to a point where no activity was detected in pNp palmitate. Initial rates of hydrolysis were calculated from the graph by measuring the slope of the increase of product formed at the beginning of the reaction. Enzyme activity was measured spectrophotometrically by following the formation of 4-nitrophenol at 410nm wavelength. The reaction mixture contained the buffer, crude enzyme extract and the substrate to a final volume of 1 ml. Twenty seconds after the substrate was added, the spectrophotometer was blanked using the reaction mixture. Increase in absorbance was measured for 10 minutes.

4-Nitrophenyl substrates tend to break down spontaneously at pH>7 therefore a control reaction was set up to be monitored together with the crude enzyme extract reaction. The linear slope resulting from the spontaneous decomposition was then subtracted from the crude enzyme reaction to avoid false positive activity. A standard curve using 4-nitrophenol was used to calculate the extinction coefficient with the R^2 value of 0.9903. One Unit (U) of activity is defined as the amount of enzyme catalysing the formation of 1 µmol of 4-nitrophenol per minute from each different substrate under the test conditions.

Activity = ((Change in OD_{410nm})/time)/ (extinction coefficient x path length)) x ((total reaction volume)/(volume of enzyme added))

Table 4.3: Activities (U/ml) of enzymes using 0.1 mM substrate

Identity	**Substrates**				
	pNp Acetate	**pNp Ferulate**	**pNp caprylate**	**pNp laurate**	**pNp palmitate**
Est1	4.95×10^{-2}	4.87×10^{-3}	4.17×10^{-3}	1.52×10^{-4}	ND
Est2	1.38×10^{-2}	1.09×10^{-3}	3.96×10^{-3}	1.28×10^{-4}	ND
Est3	2.31×10^{-2}	4.84×10^{-3}	5.06×10^{-3}	5.44×10^{-5}	ND
Est4	4.15×10^{-2}	4.53×10^{-4}	4.36×10^{-3}	9.19×10^{-4}	ND

Est5	1.86×10^{-2}	1.47×10^{-3}	4.24×10^{-3}	1.76×10^{-4}	ND
Est6	3.62×10^{-2}	6.54×10^{-4}	5.08×10^{-3}	6.69×10^{-5}	ND
Est7	2.54×10^{-2}	3.46×10^{-4}	4.67×10^{-3}	1.94×10^{-4}	ND
Est8	2.01×10^{-2}	2.3×10^{-3}	4.82×10^{-3}	4.17×10^{-4}	ND
Est9	3.48×10^{-2}	4.23×10^{-4}	4.52×10^{-3}	1.01×10^{-4}	ND
Est10	2.62×10^{-2}	3.96×10^{-4}	4.05×10^{-3}	8.47×10^{-4}	ND
Est11	5.98×10^{-2}	4.08×10^{-4}	4.44×10^{-3}	1.53×10^{-4}	ND
Est12	7.31×10^{-2}	1.44×10^{-4}	5.46×10^{-3}	1.98×10^{-4}	ND
Est13	3.44×10^{-2}	4.36×10^{-4}	5.38×10^{-3}	1.16×10^{-4}	ND
Est14	3.49×10^{-2}	5.23×10^{-4}	5.6210^{-3}	1.78×10^{-4}	ND

*ND- No activity was detected.

4.5. Substrate specificities

Hydrolytic activities of the esterases were calculated against the 4-nitrophenyl esters (acetate, C2, Caprylate, C8, Laurate, C12, and Palmitate, C16). A strong preference towards short length to medium length acyl chains was observed (C2>C8>C12>C16). Very low activity was observed in 4-nitrophenyl laurate and no activity was detected on the 4-nitrophenyl palmitate.

4.6. Biocatalysis

4.6.1 Enzyme screening

Screening for active enzymes to be used in biocatalysis was done using α-naphthyl butyrate (Figure 4.6) and naphthol AS-D chloroacetate (Figure 4.8) and these reactions were expected to have a colour change from blue to purple if the enzymes were able to hydrolyse the two substrates. Naphthol AS-D chloroacetate and α-naphthylbutyrate were used for the screening because they are bulky compounds and in this way are similar to the compounds that we aimed at resolving. In the reaction with naphthol AS-D chloroacetate, esterases enzymatically hydrolyse the substrate liberating a free naphthol compound. This compound then couples with a diazonium compound forming highly coloured deposits at the active site of the enzyme. This is what brings the colour change to the reaction mixture showing that the enzymes were active in solution. The Figures 4.7 a) and b) show the colour changes for the

reactions with α-naphthylbutyrate as compared to the control reaction which is in the tube at the top of the picture. The control tubes remained with an almost clear solution and the reactions where the enzymes were added had a deep purple colour against light. The esterase reactions shown in Figure 4.7 a) were run alongside reactions with lipases Figure 4.7 b) that were suspected to be inactive and this was confirmed as seen in Table 4.4. Figures 4.9 a) and 4.9 b) show the activity reactions using naphthol AS-D chloroacetate for esterases and lipases respectively and the activity of the enzymes was determined by comparing the colour of the reaction tubes with enzymes against the control tubes. Table 4.4 shows a summary of all the enzymes screened for activity and the colour intensities of each tube determined how active the enzymes were.

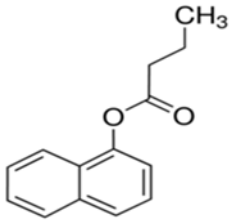


Figure 4.6: α- naphthyl butyrate chemical structure.

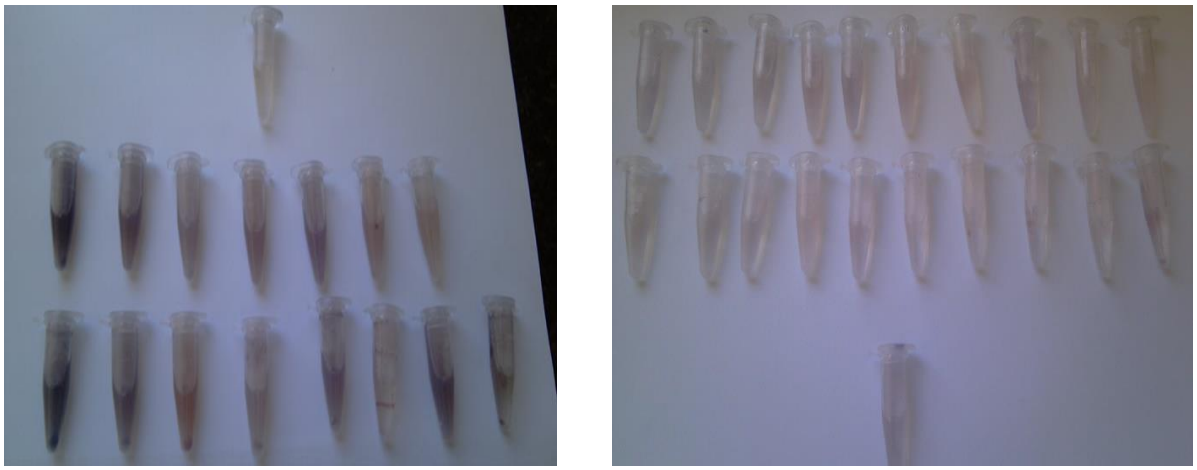


Figure 4.7:

a)Reactions using esterases on α- naphthyl butyrate b) Reactions using lipases on α- naphthyl butyrate

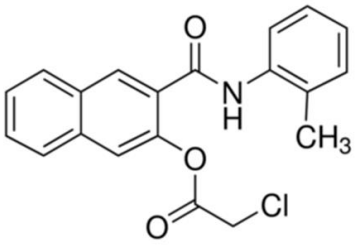


Figure 4.8: Naphthol AS-D Chloroacetate chemical structure.

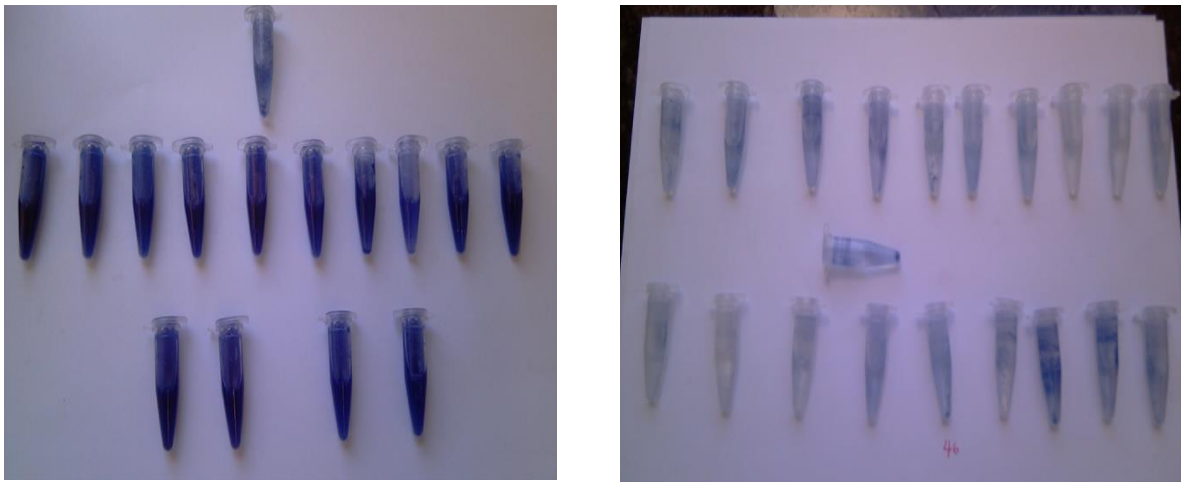


Figure 4.9:

a)Reactions using esterases on naphthol AS-D chloroacetate b) Reactions using lipases on naphthol AS-D chloroacetate

Table 4.4: Results of the esterases and lipases screening for activity for biocatalysis.

Enzyme name	Identification	α-naphthylbutyrate	Naphthol AS-D chloroacetate
Esterase 1	1	+++	+++
Esterase 2	2	+++	+++
Esterase 3	3	+++	+++
Esterase 4	4	+++	+++
Esterase 5	5	+++	+++
Esterase 6	6	+++	+++
Esterase 7	7	+++	+++
Esterase 8	8	+++	+++
Esterase 9	9	+++	+++
Esterase 10	10	+++	+++

Esterase 11	11	+++	+++
Esterase 12	12	+++	+++
Esterase 13	13	+++	+++
Esterase 14	14	+++	+++
Lipase pancreatic porcine	15	-	-
Lipase a/l “Amano” Lot#205 ILAKW 1150	16	-	-
Nagase enzymes lipase A-10FG	17	-	-
Lipozyme TL IM Novozymes	18	-	-
Lipase LIISP biocatalysis Baten 1768981	19	-	-
Lipase *C. antarctica* type B, Iyo	20	-	-
Recombinant biocatalysis ESL-001-01 (129g) with stabilizer	21	-	-
Lipase AH-D “Amano”	22	-	-
Lipase A/L “Amano” 20g lot # II AKKO26094	23	-	-
Lipase A5 Amano (8) *A.niger* LAW035145 60g Amano	24	-	-
Lipase hog pancrease 23.3µ/mg (62300.Biochemika)	25	-	-
Lipopan FBG Novozymes	26	-	-
Lipase F-AF15 50g LFW02523(10) Amano pharmaceutical	27	-	-
Lipase *Aspergillus oryzae* (951B4)250mg Biochemika	28	-	-
XP- 415 Biozymes -5	29		-
Lipase A/L –D “Amano”III 305 lot # ILAKXO2509K	30	-	-
Lipase AK-D “Amano”II 26g lot # ILAKXWZON	31	-	-
Lipase AK “Amano”	32	-	-
Recombinant biocatalysis Cat #ESL	33	-	+

003-01 1.0g Lot # 620248(1)			
Recombinant biocatalysis Cat #ESL 003-01 1.0g Lot # 620248(2)	34	-	+
Recombinant biocatalysis Cat #ESL 003-01 1.0g Lot # 620248(3)	35	-	+
Lipase A/L "Amano" 20g Lot # LAKY02570	36	-	+
Biocatalysis Lipase Lot36p 143971	37	-	+
Recombinant biocatalysis Cat #ESL 003-01 1.0g Lot # 6y0240	38	-	+
Lipase Sigma (EC: 3.1.10) Type II Crude from Porcine pancreas 9001-62-2-1	39	-	-
Recombinant biocatalysis (ESL-001-01) 21G LOT # 720268	40	-	+
Lipase AK "Amano"20 100g lot # LAKW09504	41	-	-
Lipase AY Amano IAYT02510	42	-	-
Amano lipase AK Lot # LAK 0351202	43	-	+
Lot # ESL 001-01 Lot # 6Y0240	44	-	+
Novozyme ESF LCG0600A	45	-	-
Lipase 100T	46	-	-
Lipozyme RM IM Lot 00111 (25)	47	-	-
Lipase 100T Batch # LA91231 12/4/95	48	-	+
Lipase from *Rhizopus riveus* 1.241mg (62310 Biochemika)	49	-	-
Lipase Amano LNW 03503	50	-	-
Novozyme 435 LC 200217	51	-	-
Lipo max Lot 1.00	52	-	-
Lipase from *Muco javanicus* 3.40mg (62304 Biochemika)	53	-	-
Lipase from *Muco javanicus* 9.901mg	54	-	-

(62304 Biochemika)			
Lipase AK-I 50G LOT # FLAKY0452/02K	55	-	-
Lipase AK-675 #54-001	56	-	-
Novozyme 525F LCG06078	57	-	-
Novozyme 308 Lot 340014	58	-	-
SP398 LAN 00052	59	-	-
Novo 388	60	-	-
Novozyme 388 (1)	61	-	-
Novozyme 388 catalogue LON 00014	62	-	-
Novozyme 388 (L)	63	-	-
Novozyme 388 LON 00020	64	-	-
Altalase (Novo) 2.4mg AMM 5022	65	-	-
Alcalase 24C FG	66	-	-
Lipase from *Candida rugosa* 62316-1-60kc	67	-	-
Lipase from wheat germ 62306-500mg-kc-f	68	-	-
Lipase from *Rhizopus arrhizus* 62305-10-kc	69	-	-
Lipoprotein lipase from *Burkholderia spp* 62336-50mg-kc-f	70	-	+
Lipase from *Aspergillus spp* 84205-100mg kc	71	-	-
Lipase from *Mucor javanicus* 62304-500mg-kc-f	72	-	-
Lipase from *Penicillium camemberti* 96888-500mg kc-f	73	-	-
Lipase from *Aspergillus oryzae* 62285-100mg kc-f	74	+++	+++
Lipoprotein lipase from *Pseudomonas spp* 62335-10mg-kc (small)	75	-	-
Lipase from *Candida rugosa* 90860-	76	-	+

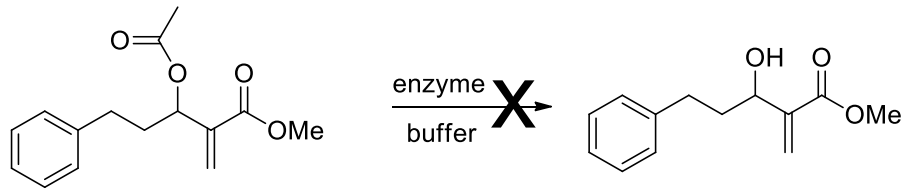
500mg-kc			
Lipase B *Candida Antarctica* recombinant from *Aspergillus oryzae* (62288-50mg)	77	-	-
Lipase from *Pseudomonas fluorescence* 28602-50mg-kc	78	-	+
Lipase from *Pseudomonas cepacia* 62309-100mg-kc	79	-	+
Lipase from *Rhizopus oryzae* 80612-500mg-kc	80	-	-
Lipase from *Rhizopus riveus* 62310-10mg-kc	81	-	-
Lipase from *Candida Antarctica* 65986-100mg-kc	82	-	-
Lipase from *Pseudomonas spp* 95608-50mg-kc	83	-	-
Lipase from Porcine pancrease L3126-10mg-kc	84	-	-
Lipase from *Mucor miehei*	85	-	+
C. rugosa Lipase AK "Amano"20g LAYA0750964G	86	-	-
Novozyme 435-LC200233	87	-	-

+++ High activity, + low activity, - no activity

4.6.2. Enzymatic resolution of enantiomers synthesised using the Morita-Baylis-Hillman reaction.

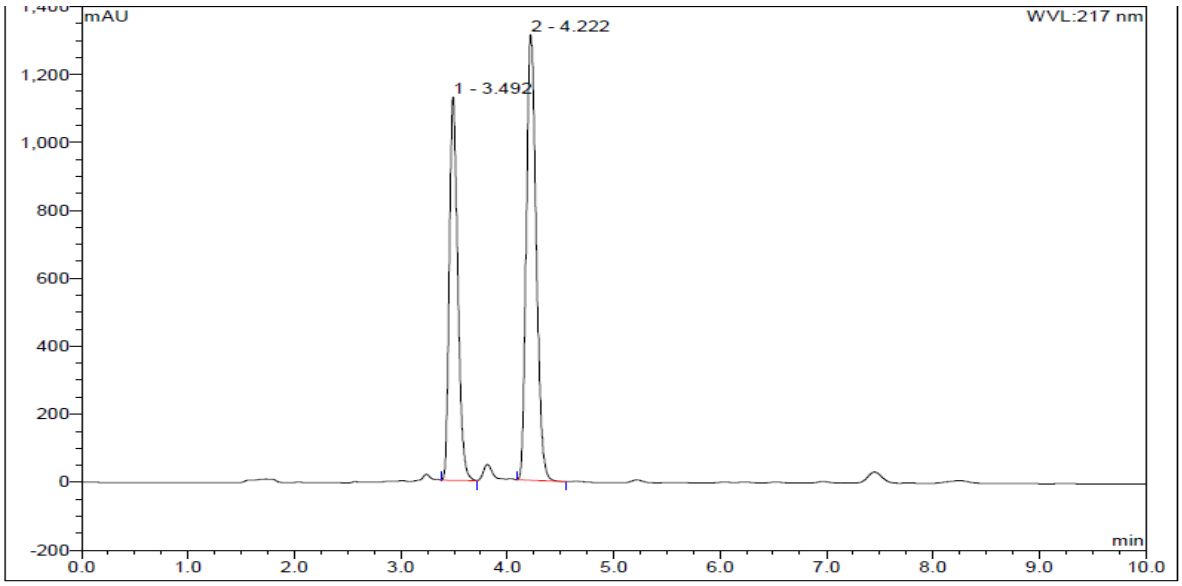
One of the main objectives of this study was to be able to use the overexpressed esterases in hydrolysis or modification of chemical compounds in the synthesis of new drugs. After screening the enzymes, the active enzymes were now used in biocatalysis of compounds that will be used as intermediates in the synthesis of potential HIV-1 protease inhibitors. The first attempt was on compound 1a (methyl 3-acetoxy-2-methylene-5-phenylpentanoate) which

was synthesised as a racemic mixture. Enzymatic hydrolysis of compound 1a was attempted following procedures reported by Hayashi *et al*. (1998), using lipases and esterases that showed high activity during the screening reaction. 20 mg of compound 1a dissolved in 5 ml of acetone was added to 10 mg of lipase in a 5ml sodium phosphate buffer at pH 7.0. For esterases 800 µl of the 100 mM sodium phosphate buffer, pH 7.0 was added to 100 µl of enzyme and the reaction was started by adding 100 µl of the substrate. The reactions were left at 40°C for 48 hours and then the results were analysed using HPLC. Standards for the starting material and desired product were run first and the starting material peak eluted at 4.222 min on HPLC and the desired product peak eluted at 3.492 min as shown in Figure 4.10.



Compound 1a **Compound 1b**

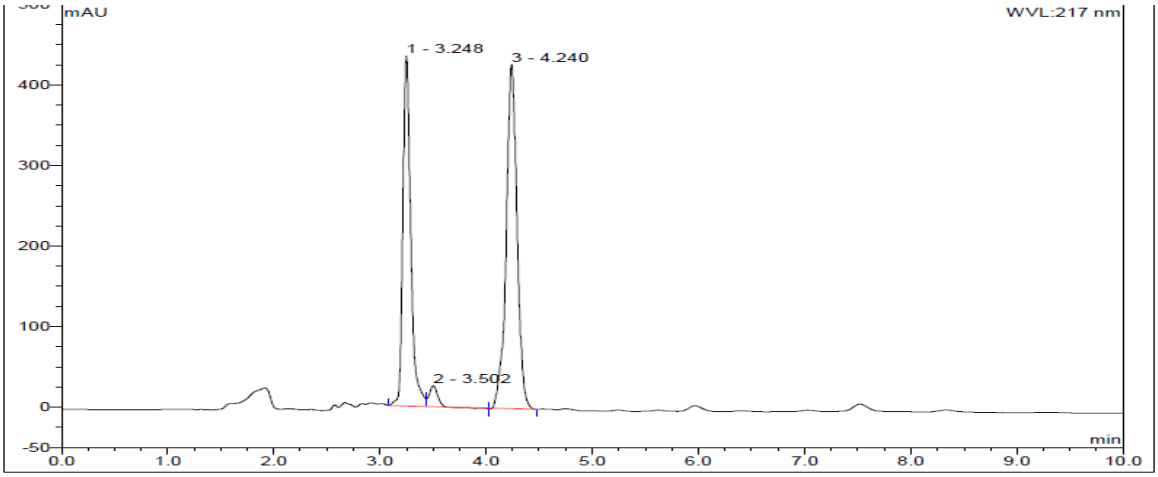
Scheme 4.1: Enzymatic hydrolysis of Compound 1a.



No.	Ret.Time min	Peak Name	Height mAU	Area mAU*min	Rel.Area %	Amount n.a.	Type
1	3.49	n.a.	1127.931	104.416	42.53	n.a.	BMB*
2	4.22	n.a.	1311.686	141.109	57.47	n.a.	BMB*
Total:			2439.617	245.525	100.00	0.000	

Figure 4.10: HPLC elution times for compounds 1a and 1b.

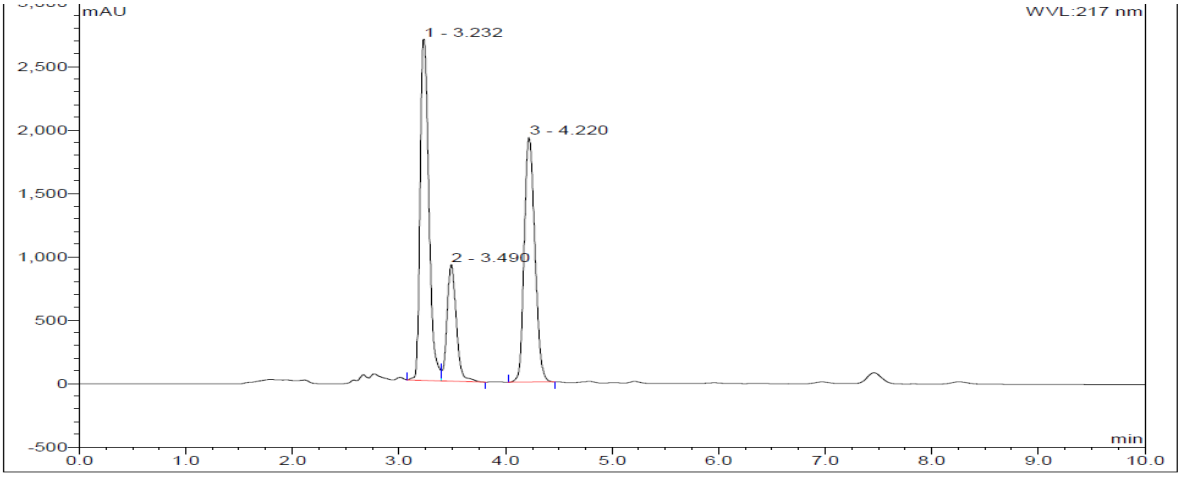
For hydrolysis reactions, the results after 48 hours showed that in the control reaction which was run under the same conditions without the enzyme, the starting material was still present at peak 4.240 min and the desired material had a very small peak at peak 3.502 min. There was formation of a new peak from an unknown compound at peak 3.248 min as shown in the HPLC results in Figure 4.11.



No.	Ret.Time min	Peak Name	Height mAU	Area mAU*min	Rel.Area %	Amount n.a.	Type
1	3.25	n.a.	435.052	37.846	41.71	n.a.	BM *
2	3.50	n.a.	26.039	2.285	2.52	n.a.	M *
3	4.24	n.a.	427.410	50.606	55.77	n.a.	MB*
Total:			888.501	90.737	100.00	0.000	

Figure 4.11: HPLC results of the control reaction.

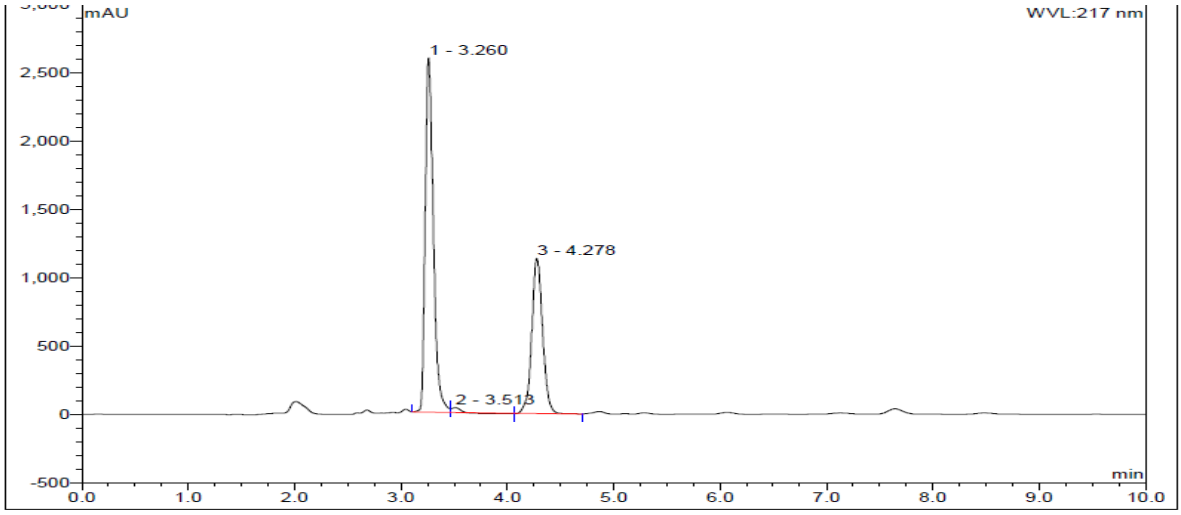
Results from hydrolysis using esterases showed almost the same results as the control reaction with the new peak of the unknown compound at 3.232 min, the desired compound at 3.490 min and also the starting material at 4.220 min. The only difference was the relative area of the desired compound which had increased from 2.52% in the control to 15.97% in the esterase reaction. Figure 4.12 shows results for Est 1 but most of the esterases did not show any hydrolysis of compound 1a. Table 4.5 below shows a summary of the enzyme results after hydrolysis which was put in terms of the relative areas of peaks of the compounds formed during the reaction in percentages.



No.	Ret.Time min	Peak Name	Height mAU	Area mAU*min	Rel.Area %	Amount n.a.	Type
1	3.23	n.a.	2692.867	259.358	45.06	n.a.	BM *
2	3.49	n.a.	918.521	91.937	15.97	n.a.	MB*
3	4.22	n.a.	1929.551	224.247	38.96	n.a.	BMB*
Total:			5540.939	575.542	100.00	0.000	

Figure 4.12: Results after enzymatic resolution of compound 1a using Est1.

For the lipase reactions most of the enzymes showed no hydrolysis and Lipase 18 had results that were similar to the control reaction with the new peak at 3.260 min, the desired product at 3.51 min and the starting material at 4.278 min. The desired product had the relative area of 0.36% which was very low and not significant as shown in Figure 4.13.



No.	Ret.Time min	Peak Name	Height mAU	Area mAU*min	Rel.Area %	Amount n.a.	Type
1	3.26	n.a.	2595.181	230.890	63.04	n.a.	BM *
2	3.51	n.a.	36.395	1.321	0.36	n.a.	M *
3	4.28	n.a.	1135.801	134.073	36.60	n.a.	MB*
Total:			3767.377	366.284	100.00	0.000	

Figure 4.13: Results after enzymatic resolution of compound 1 using Lipase enzyme 18.

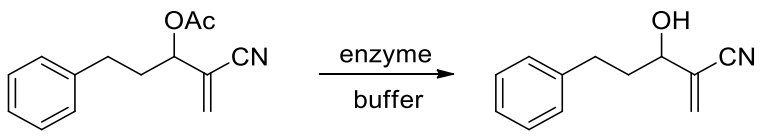
Table 4.5: A summary of the 36 enzymes tested for resolution by hydrolysis.

Enzyme number	Conversion to expected product	Relative area % of starting material	Relative area % of desired product	Relative area of unknown compound	Relative area of other new peaks
1	Yes	38.96	15.97	45.06	
2	Yes	26.32	34.44	39.24	
3	No	43.02	-	56.98	
4	Yes	-	47.77	52.23	
5	Yes	7.22	81.44	11.34	
6	Yes	30.22	2.49	67.29	
7	Yes	45.94	15.78	38.28	
8	Yes	59.53	3.47	36.45	0.55
9	No	32.58	-	67.42	
10	Yes	57.41	1.88	40.71	
11	Yes	1.40	53.24	45.36	
12	Yes	1.51	56.54	41.95	
13	Yes	31.82	0.56	67.61	
14	Yes	11.77	70.92	15.87	1.44
18	No	36.60	0.36	63.04	
20	Yes	2.98	-	54.46	42.56
21	Yes	28.60	27.87	43.53	
27	Yes	54.03	25.56	20.41	
28	Yes	31.83	1.11	67.06	
30	Yes	72.71	1.15	25.49	0.56
33	Yes	2.01	49.49	48.50	
34	Yes	18.10	56.61	25.29	
35	Yes	12.98	78.48	8.54	
36	No	20.39	-	79.61	
37	Yes	19.99	16.75	63.27	
38	Yes	7.07	47.61	45.32	

40	Yes	5.95	48.54	45.50	
41	No	26.52	-	73.48	
43	No	23.87	-	76.13	
44	Yes	5.61	37.02	57.37	
46	Yes	94.64	2.87	2.49	
48	No	25.12	-	74.88	
70	Yes	26.67	34.34	38.99	
74	No	43.15	-	56.85	
78	No	27.41	-	72.59	
85	No	17.74	-	82.26	
Control1	Yes	40.82	1.57	57.61	
Control2	Yes	55.77	2.52	41.71	

The desired results from hydrolysis of compound 1a were that 50% of the starting material be converted to product. This would result from hydrolysis of one of the two enantiomers which would form a peak at 3.5 min. All the results were disappointing, as even the control reaction showed hydrolysis of the starting material to unknown compounds which made it difficult to pursue this approach for the resolution of the two enantiomers of compound 1a. These experiments could have been complicated by the fact that the compound being hydrolysed has 2 esters and the enzymes are capable of hydrolysing either or both of the 2 esters. I did not pursue resolution of compound 1a because of this reason.

A second compound was chosen for the investigation of enzymatic resolution, as shown in Scheme 4.2 using all the selected lipases and the 14 esterases. Control reactions were run alongside to prevent false positive results and for comparison. Firstly in order to find the best buffer to work with we tested different buffers so that there would be no autohydrolysis during the reaction. The results for the different buffers tested are summarised in Table 4.6. The compound proved to be stable in many buffers as all the HPLC results showed no formation of new compounds in the absence of enzymes or any autohydrolysis.



Compound 2 **Compound 3**

Scheme 4.2: Enzymatic hydrolysis of Compound 2.

Table 4.6: Summary of different buffers tested for autohydrolysis of compound 2

Conditions tested	After 2 hours	After 7 hours	After 24 hours	After 48 hours
Compound 2 in 5% acetone phosphate buffer	-	-	-	-
Compound 2 in 10% acetone phosphate buffer	-	-	-	-
Compound 2 in acetone phosphate (1:2.5)	-	-	-	-
Compound 2 in 5% acetone tris buffer	Tris peak detected	Tris peak detected	Tris peak detected	-
Compound 2 in 10% acetone tris buffer	-	Tris peak detected	Tris peak detected	-
Compound 2 in 5% DMSO phosphate buffer	DMSO peak detected	DMSO peak detected	-	-
Compound 2 in 10% DMSO phosphate buffer	DMSO peak detected	DMSO peak detected	DMSO peak detected	-
Compound 2 in 5% DMSO tris buffer	-	DMSO peak detected	DMSO peak detected	-
Compound 2 in 10% DMSO tris buffer	DMSO peak detected	DMSO peak detected	DMSO peak detected	-
Compound 2 in acetone only	-	-	-	-
Compound 2 in water only	-	-	-	-
Compound 2 in tris only	Tris peak detected	Tris peak detected	Tris peak detected	-
Compound 2 in potassium phosphate buffer only	-	-	-	-
Sodium Phosphate buffer only	-	-	-	-
Tris buffer only	-	-	-	-

- Only starting material detected

The buffer chosen to conduct hydrolysis experiments was potassium phosphate 50 mM at pH 7.2. 52 mg of the cyano derivative compound 2 (2-cyano-5-phenylpent-1-en-3-yl acetate) was added to 750 µl of acetone. 50 µl of this was then taken and mixed with 850 µl of the potassium phosphate buffer and 100 µl of enzyme. The reactions were conducted at 20 °C and results were checked at different times. The results are summarised in Table 4.7. Reactions were followed using TLC and most of the enzymes did not show any hydrolysis, therefore only results from a few selected enzymes that had promising results are summarised in Table 4.7. Column 1 of the table shows the relative areas from the HPLC analysis of the starting material (compound 2) and the desired product formed after hydrolysis (Compound 3 (3-hydroxy-2-methylene-5-phenylpentanenitrile)). Very low conversion rates were observed for any of the reactions therefore the reaction conditions were changed. The temperature was increased to 40°C and chiral HPLC analysis was conducted after 24 and 48 hours as summarised in Table 4.7 (Column 2 and 3).

Table 4.7: Results of hydrolysis at various time intervals.

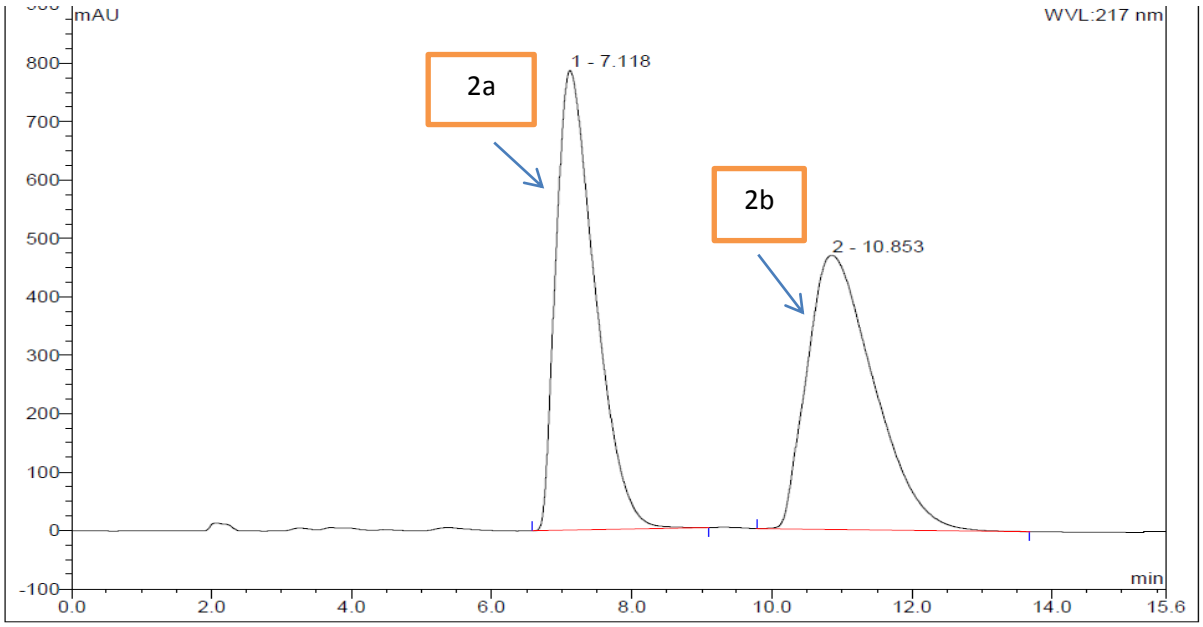
	*	+	+
Enzyme number	Relative areas of compound 2 and compound 3 (30 minutes, 20°C)	Relative areas of enantiomers 2a and 2b after 24 hours, 40°C	Relative areas of enantiomers 2a and 2b after 48 hours, 40°C
Est 4	-	-	41.22:20.18
Est 12	-	-	35.55: 15.23
21	98.93:1.07	-	-
33	91.53:8.57	-	-
34	100:00	-	-
35	98.58:1.42	-	-
38	96.03:3.97	-	-
40	90.69:9.31	-	-
44	99.73:0.27	-	-
70	98.00:2.00	-	-
20	-	49.44:50.56	59.02:40.98
36	-	43.43:56.57	60.17:39.83
37	-	54.00:46.00	62.45:37.55

78	-	75.22:24.78	97.51:2.49
Buffer only	-	-	-
Acetone	-	-	-

* From C-18 HPLC

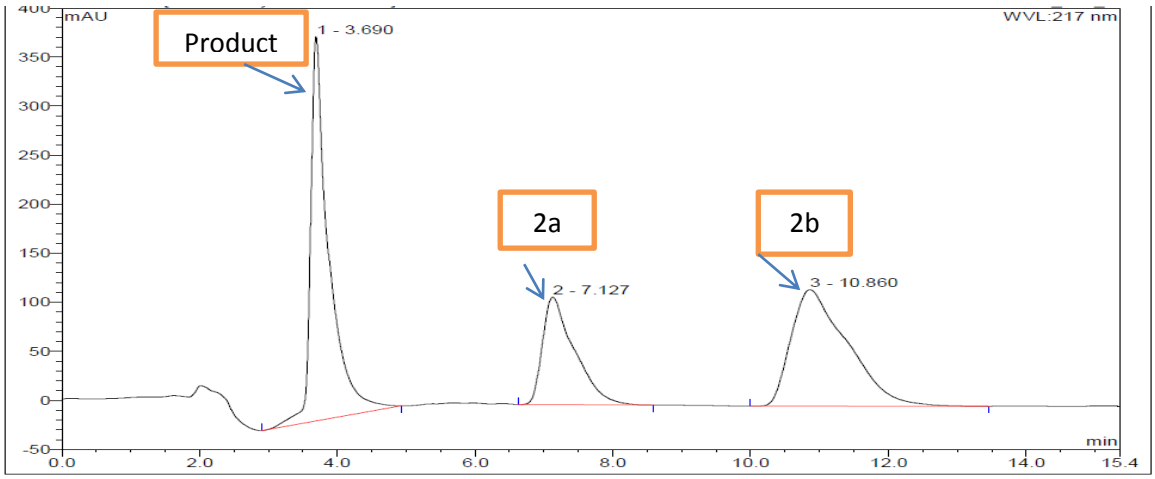
+ From chiral HPLC

For chiral HPLC analysis, the two enantiomers of the starting material appeared at 7.12 min (2a) and 10.85 min (2b) (Figure 4.14). Disappearance of the starting material peaks was monitored. For enantioselective reactions, one of the two peaks will disappear faster than the other and thus a difference in relative peak size is an indication of an enantioselective enzyme. In Figure 4.15 Est4 was used and the results showed partial selectivity in the hydrolysis of the two compounds. The relative areas showed a ratio of 1:2 compared to the 1:1 ratio at the beginning of the reaction. The peak seen at 3.690 min is a mixture of both enantiomers of the expected product. Figure 4.16 shows results after hydrolysis using lipase enzyme 20. One enantiomer was completely hydrolysed and the other remained. These were good results as they indicated that a completely enantioselective hydrolysis had occurred.



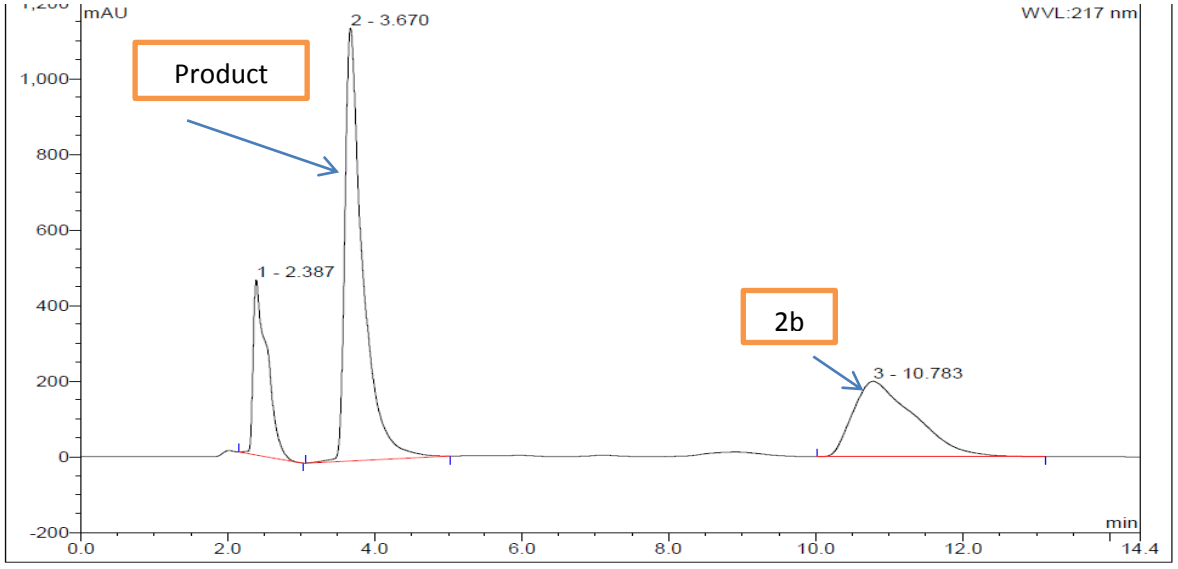
No.	Ret.Time min	Peak Name	Height mAU	Area mAU*min	Rel.Area %	Amount	Type
1	7.12	n.a.	786.885	508.864	49.57	n.a.	BMB
2	10.85	n.a.	469.648	517.735	50.43	n.a.	BMB
Total:			1256.533	1026.599	100.00	0.000	

Figure 4.14: HPLC elution times for the two enantiomers of compound 2 (2a and 2b)



No.	Ret.Time min	Peak Name	Height mAU	Area mAU*min	Rel.Area %	Amount n.a.	Type
1	3.69	n.a.	391.948	120.085	41.22	n.a.	BMB*
2	7.13	n.a.	109.647	58.801	20.18	n.a.	BMB*
3	10.86	n.a.	118.770	112.439	38.60	n.a.	BMB*
Total:			620.365	291.326	100.00	0.000	

Figure 4.15: Results from hydrolysis of compound 2 using Est4.



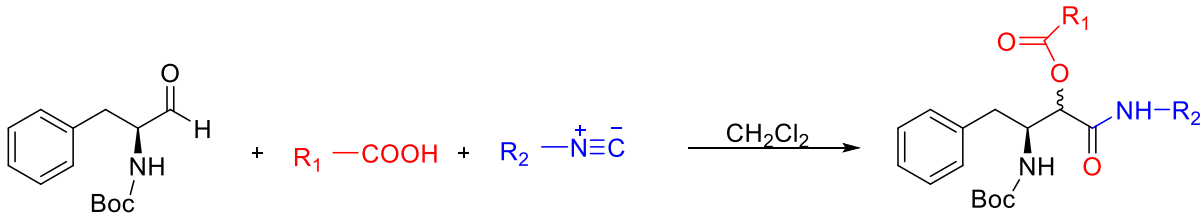
No.	Ret.Time min	Peak Name	Height mAU	Area mAU*min	Rel.Area %	Amount n.a.	Type
1	2.39	n.a.	463.507	102.313	16.63	n.a.	BMB*
2	3.67	n.a.	1144.099	321.835	52.31	n.a.	BMB*
3	10.78	n.a.	198.720	191.070	31.06	n.a.	BMB*
Total:			1806.326	615.217	100.00	0.000	

Figure 4.16: Results from hydrolysis of compound 2 using Lipase 20.

4.6.3 Enzymatic resolution of diastereomers synthesised using the Passerini reaction.

Another attempt at selective enzymatic hydrolysis of compounds was performed on compounds synthesised using the Passerini Amine Deprotection Acyl Migration (PADAM)

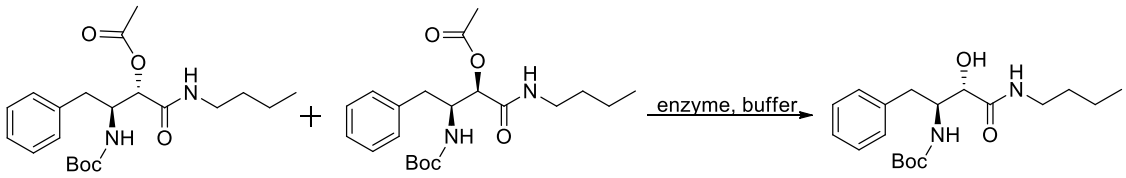
reaction. Scheme 4.3 shows the reaction used to synthesise compound 4 which was a mixture of diastereomers. Compound 4 was a peptidomimetic compound. Peptidomimetic compounds are peptide-like molecules, but with modifications that have advantages such as increased stability and biological function.



Compound 4

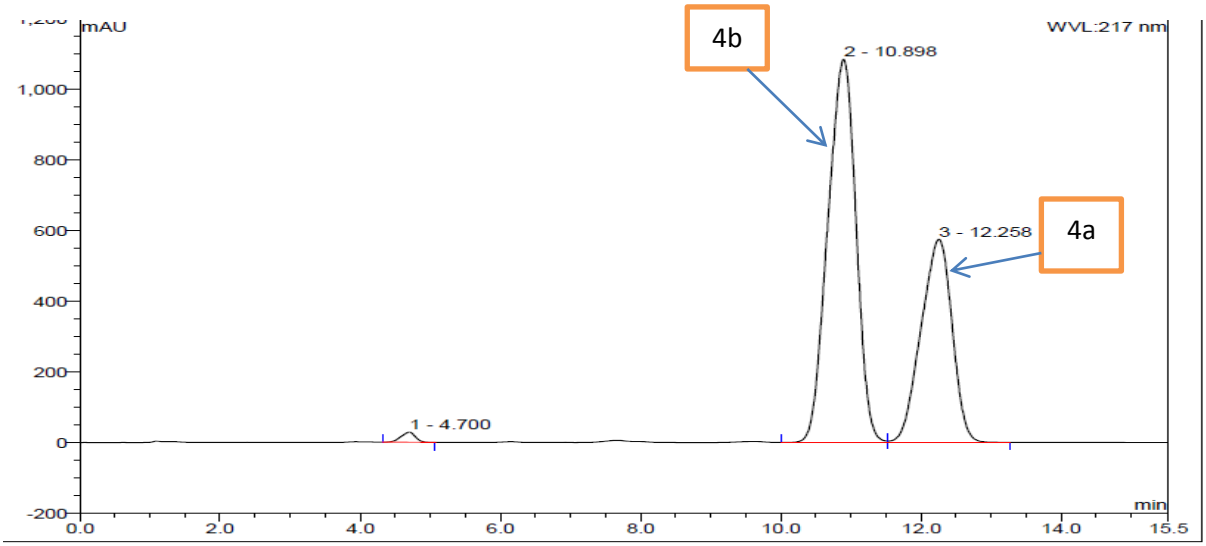
Scheme 4.3: Passerini reaction

Compound 4 was prepared as a mixture of diastereomers in a 1.7:1 ratio and the overexpressed esterases were used to hydrolyse diastereomers 4a ((2*S*,3*S*)-3-((*tert*-butoxycarbonyl)amino)-1-(butylamino)-1-oxo-4-phenylbutan-2-yl acetate) and 4b ((2*R*,3*S*)-3-((*tert*-butoxycarbonyl)amino)-1-(butylamino)-1-oxo-4-phenylbutan-2-yl acetate) (Scheme 4.4). Figure 4.17 shows the standard HPLC results from HPLC analysis of the two diastereomers (compound 4a and compound 4b). The reactions were set at 40 °C after mixing 800 µl of sodium phosphate buffer at pH 7.5 with 100 µl of enzyme and 100 µl (60 mg/ml) of substrate. The reaction was shaken for 48 hours and was then checked using HPLC analysis. Only 3 esterases were able to perform the hydrolysis and these were Est1, Est11 and Est12 as shown in Figure 4.18, 4.19 and 4.20 respectively. Est1 increased the diastereomeric ratio to 2.3:1, Est11 showed very slight selectivity as it had a 1.8:1 ratio after the reaction. Selectivity in hydrolysis was mainly observed on the reaction with Est12 as it increased the diastereomeric ratio to 3.2:1 compared to the 1.7:1 at the beginning of the reaction. The results showed preferential conversion of compound 4a to form compound 5a (*tert*-butyl ((2*S*,3*S*)-4-(butylamino)-3-hydroxy-4-oxo-1-phenylbutan-2-yl)carbamate under the reaction conditions. The alcohol product appears as a peak at 7.97 min under those conditions. Further research on this enzyme for selective hydrolysis is being pursued.



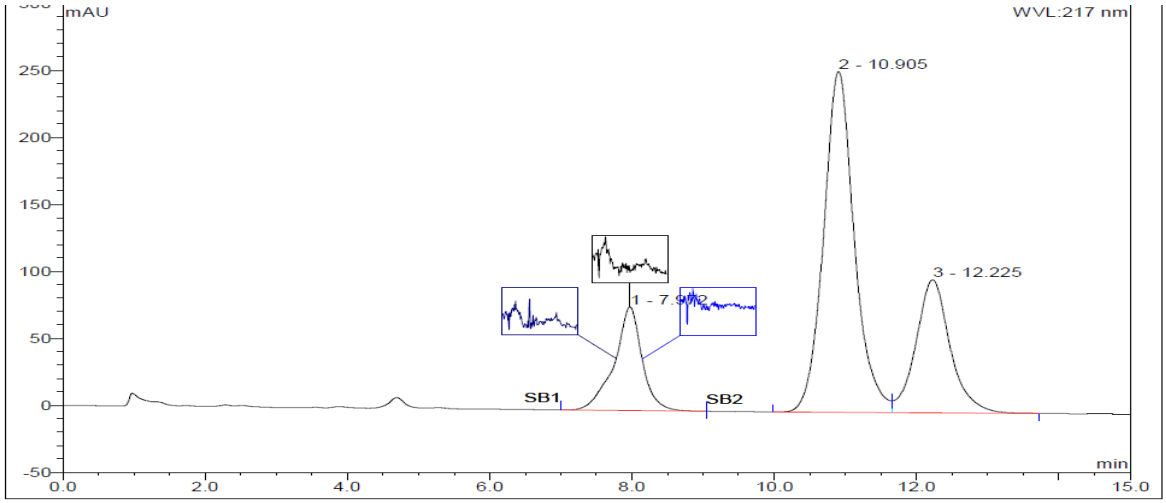
Compound 4a **Compound 4b** **Compound 5a**

Scheme 4.4: Enzymatic hydrolysis of **Compound 4**



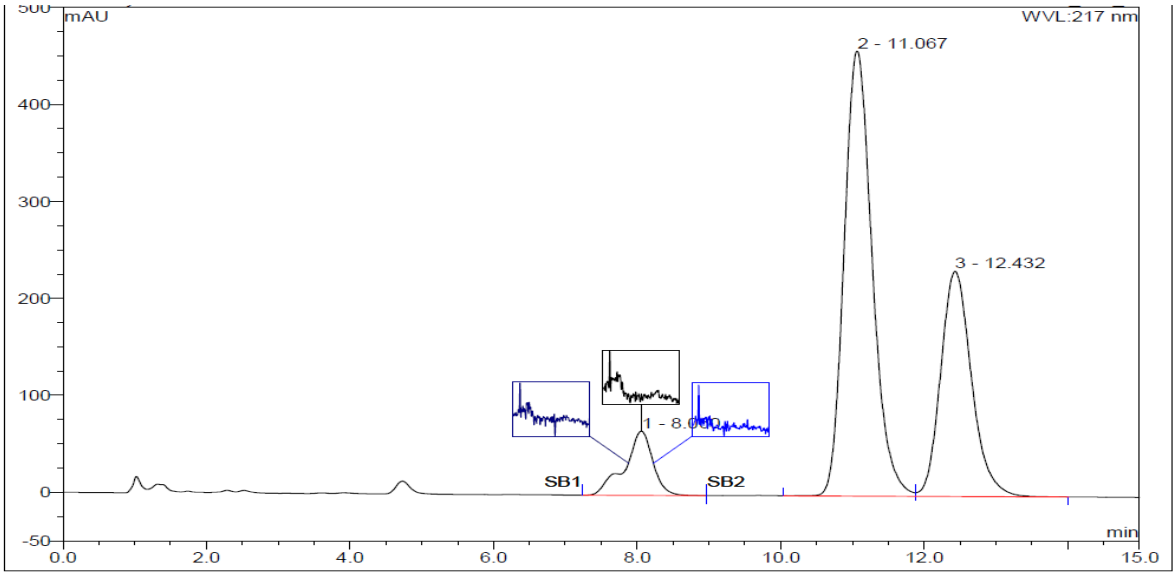
No.	Ret.Time min	Peak Name	Height mAU	Area mAU*min	Rel.Area %	Amount	Type
1	4.70	n.a.	28.662	6.753	0.83	n.a.	BMB
2	10.90	n.a.	1084.580	507.435	62.67	n.a.	BM
3	12.26	n.a.	575.223	295.474	36.49	n.a.	MB
Total:			1688.466	809.663	100.00	0.000	

Figure 4.17: HPLC elution times for compound 4a and compound 4b



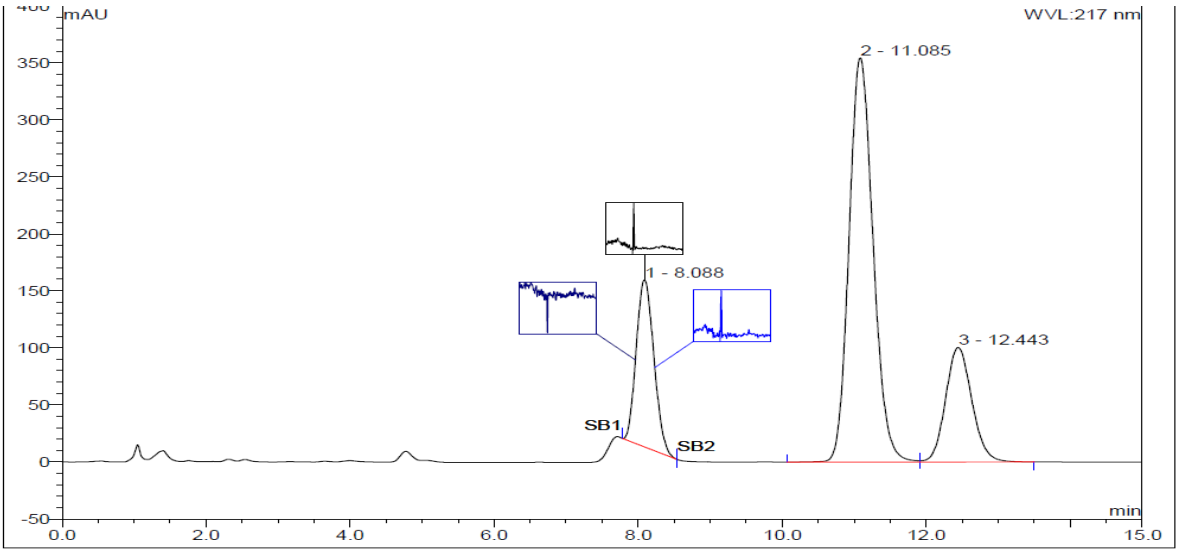
No.	Ret.Time min	Peak Name	Height mAU	Area mAU*min	Rel.Area %	Amount	Type
1	7.97	n.a.	77.346	35.055	16.09	n.a.	BMB
2	10.91	n.a.	254.218	127.408	58.47	n.a.	BM
3	12.23	n.a.	99.153	55.441	25.44	n.a.	MB
Total:			430.716	217.904	100.00	0.000	

Figure 4.18: Results of hydrolysis of compound 4a and compound 4b using Est1.



No.	Ret.Time min	Peak Name	Height mAU	Area mAU*min	Rel.Area %	Amount	Type
1	8.06	n.a.	66.314	30.293	8.55	n.a.	BMB
2	11.07	n.a.	458.828	208.075	58.74	n.a.	BM
3	12.43	n.a.	232.350	115.892	32.71	n.a.	MB
Total:			757.491	354.259	100.00	0.000	

Figure 4.19: Results of hydrolysis of compound 4a and compound 4b using Est11.



No.	Ret.Time min	Peak Name	Height mAU	Area mAU*min	Rel.Area %	Amount	Type
1	8.09	n.a.	146.349	41.654	18.78	n.a.	BMB
2	11.09	n.a.	354.532	137.570	62.04	n.a.	BM
3	12.44	n.a.	100.443	42.537	19.18	n.a.	MB
Total:			601.324	221.761	100.00	0.000	

Figure 4.20: Results of hydrolysis of compound 4a and compound 4b using Est12.

CHAPTER 5: DISCUSSION AND CONCLUSION

5.1. General esterase properties.

Esterases are enzymes that are subdivided into various categories. These divisions are based on the characteristics of the enzymes and the primary structures as well as the gene sequences (Arpigny *et al*., 1999). The 14 different genes of esterases that we transformed and subsequently expressed show differences in gene sequences and also differences in functions of the proteins from these genes. The genes had various base pair lengths ranging from 783 base pairs (bp) to 1545 bp (table 4.1). The confirmation of the gene sizes was done by sequencing at Inqaba Biotech (South Africa) followed by use of the DNA Baser to clean up the sequences and Blast to match the sequences in the existing database. This information confirmed that all the plasmids received from CSIR Biosciences contained different genes but they were all esterase genes. Culture based techniques have been used for a long time to discover new genes but they are limited. The new culture-independent techniques were developed for access to a wide range of novel esterase genes that can be put into various industrial applications (Lorenz *et al*., 2002). Sequence based screening approaches allow rapid cloning and expression of genes and genes can be accessed directly from community DNA in the environment. In this study genes that were used were isolated using metagenomic approach and from various sources as shown in Table 4.1.

It is important that during hydrolysis of compounds, firstly the acyl chain lengths of the substrate are considered because enzymes are affected by the bulkiness of the substrates that they are working on. During hydrolysis esterases are differentiated from lipases by the preferences for their substrates. Esterases hydrolyse short-chain acyl esters whilst lipases prefer the long chain esters (Jaeger *et al*., 1999). The specificity to substrates of each carboxylesterase can be attributed to a number of factors which include the sizes of each substrate and the hydrophilicity and hydrophobicity of the substrates binding to the active site (Jaeger *et al*., 1999). The profiling of substrates in this study further confirmed that the enzymes were esterase as there was more activity in the short chain esters than in long chain esters as reported in the results section.

5.2. Expression and Biochemical characterisation of the 14 esterases.

The plasmids carrying the genes had antibiotic selectors of which in this case 13 were for ampicillin and 1 was for kanamycin. This prevented all the other cells that might contaminate

the media during expression from growing as they would be killed by the antibiotic. During expression induction of the cells was done using isopropyl β-D-1 thiogalactopyranoside (IPTG). This is a compound that triggers transcription of the *lac* operon and is used in protein expression were the gene is under control of the *lac* operator. After confirming enzyme expression it was important to check if the enzymes were active and this was done using 4-nitrophenyl acetate. The different varieties of esterases showed various degrees of activity with Est12, Est11 and Est1 being the most active of the 14 enzymes as shown in Table 4.1. The activities of these enzymes were also calculated on various substrates and generally all the enzymes preferred short chain molecules compared to long chain molecules. Long chain molecules give problems in fitting in the active site of the enzymes as they are bulky.

The biochemical characterisation of the enzymes enabled the finding of optimum conditions in which the enzymes will be able to work. It is of major importance in biocatalysis to know the optimum temperature and pH at which an enzyme can work so as to avoid denaturing the enzyme under undesired conditions and also to maximise substrate formation. Most of the enzymes showed an optimum pH of 8.0 as shown in Table 4.2. Esterases were denatured at low pH values and there was little, if any, activity under pH 4. In industry enzyme use is usually a problem as the conditions are not conducive to enzyme functioning and the enzymes quickly become denatured. Therefore, it is of utmost importance to do more research on ways of implementing enzymes in industrial applications without denaturation. The observed optimum temperatures in this study were slightly different from the ones previously reported by Rashamuse *et al*., (2014) when they worked with the same genes due to the differences in conditions in which the study was done. The temperature optima are not intrinsic enzyme properties as they are affected by various thermal properties and the duration of each assay (Daniel and Danson, 2010). However the dependence of enzyme activity on temperature is usually defined in two processes which are the catalytic reaction of the enzyme and the irreversible inactivation of the enzyme (Daniel and Danson, 2010).

Expression systems of proteins have significant importance in industrial fermentation notably in biopharmaceuticals production and in enzyme manufacture. It is important in industry to be able to use crude enzyme extract as this will cut down on the costs of purification of the enzymes at large scale. This research is important as it enabled the use of crude enzyme extracts to get some positive results on the separation of racemic mixtures of newly synthesised compounds.

5.3. Enzymatic resolution of enantiomers synthesised using the Morita-Baylis-Hillman reaction.

The first activity test we did on the enzymes for hydrolysis of bulky compounds was done using naphthol AS-D chloroacetate and α-naphthylbutyrate. The reaction mixture for both the compounds turned blue immediately after mixing together with enzyme and buffer. The reaction was left to run for 20 minutes as outlined in the materials and methods and the mixture turned from blue to purple for enzymes that were active on the compounds and results are summarised in Table 4.4.

Compounds 1a and 2 were synthesised as racemates using the Morita-Baylis-Hillman (MBH) reaction (School of Chemistry, Witwatersrand).These were intermediates in the synthesis of peptidomimetic compounds and the result was a racemic mixture. Peptidomimetic compounds are small protein-like compounds designed to mimic peptides. Usually they have alterations done removing groups on the peptides that cause low pharmacokinetic properties but do not alter the main features on the compound responsible for the biological effect (Cherry and Murphy, 2004).

The compounds of interest in this research were a mixture of enantiomers or diastereomers, but it is important to have an optically pure compound for maximum biological activity. Enzymatic kinetic resolution usually results in the conversion of 50% of the starting material and the other 50% remains as an unconverted enantiomer (Kim *et al*., 2012). Esterases are usually substrate specific and they are used in most hydrolysis reactions like in this study. In the first set of reactions for compound 1a, the starting material was seen at peak 4.22 min by HPLC analysis and if the reaction was successful the peak would be reduced by half forming a new compound at 3.40 min as shown in Figure 4.10. The starting material was a mixture of enantiomers. The enantiomers had 2 esters and either of the two could be hydrolysed by the enzymes giving complicated results. The control reaction results in Figure 4.11 show hydrolysis forming a new peak of an unknown compound at 3.248 minutes with a relative area of 41.71%. Figure 4.12 and Figure 4.13 show results from the reactions using both lipases and esterases and still the peak of the unknown compound was present. Table 4.5 shows a summary of all the tested enzymes and no significant hydrolysis occurred. Due to these complications in interpreting the results, the investigation was left and we pursued other compounds with only a single ester group.

Compounds can be hydrolysed in the absence of enzymes and thus the effect of different buffers on autohydrolysis was tested and the results are summarised in Table 4.6. This helped to choose the correct buffer for use during the reaction. Table 4.7 also showed the investigation of time together with different temperatures for the reaction to maximise the product formation. The reaction was followed by monitoring the starting material on chiral HPLC to observe if there were any changes in the relative amounts of the two enantiomers. Only the enzymes that showed promising results were discussed. Figure 4.13 shows the results after hydrolysis with Est4 and it was partially enantioselective. The relative areas of the two enantiomers were 20.18% and 38.60% after 48 hours. The conditions of this reaction could be altered and further investigated to maximise selectivity. Lipase 20 was selective as seen in Figure 4.14, one of the enantiomers was completely hydrolysed and only one remained. The remaining enantiomer was eluted at 10.78 min with 31.06% relative area. This shows that lipase 20 was successfully enantioselective as it preferred the hydrolysis of one enantiomer over the other. If column chromatography is used to separate the formed products from the remaining enantiomer, an optically pure compound will remain and allow further investigations in synthesising new potential HIV-1 protease inhibitors.

5.4. Enzymatic resolution of diastereomers synthesised using the Passerini reaction.

Diastereomers are two chiral compounds that usually have different biological activities. The separation of the diastereomers of interest in this investigation by column chromatography has previously been tested, but without success (Gravestock *et al*., 2012). There are other means of separating diastereomers like preparative HPLC. In hydrolysis of compounds during the enzymatic resolution of diastereomers both lipases and esterases can be used as they are from the same family of hydrolases. As this research had made use of lipases before for separation of diastereomers, esterases were tested for hydrolysis of the same compounds.

The aim of the investigation was to hydrolyse an ester group on one of the compounds, in a mixture of diastereomers, previously synthesised by the Passerini reaction, and remain with an optically pure compound in an attempt to synthesis potential HIV-1 protease inhibitors. The product formed after the hydrolysis should be readily separable from the unhydrolysed material by column chromatography. Based on our investigation enzymatic kinetic resolution was done using 14 different varieties of esterases.

The esterases were tested for the ability to selectively hydrolyse the ester group on the newly formed stereogenic centre of the Passerini product considering that other researchers had used the same approach to separate different compounds with success (Szymanski and Ostaszewski, 2006). Scheme 4.3 shows the Passerini reaction used in the synthesis of compound 4 which was a mixture of two diastereomers. Hydrolysis of compound 4a was preferred over 4b forming an alcohol (5a) and the reaction would allow compound 4b to remain and be separated by column chromatography forming an optically pure compound (Scheme 4.4). Figure 4.17 shows that the two diastereomers eluted after 10.898 min and 12.258 min on HPLC analysis. If one diastereomer is completely hydrolysed to form an alcohol, one of the two diastereomers would remain and they could be isolated in pure form.

Figure 4.18 shows the results after a reaction with Est1 and the enzyme showed selectivity as compound 4a and compound 4b were a 2.3:1 ratio after 48 hours. After hydrolysis using Est11 the enzyme proved to be, not significantly selective whilst Est12 was the enzyme of interest as it showed the highest selectivity among the three enzymes. This is on-going research and the reactions will now be repeated on a large scale and separation will be done to isolate single diastereomers and the compounds synthesised will be tested for biological activity as possible protease inhibitors.

5.5 Conclusion

In conclusion, the 14 esterases were overexpressed and characterised showing different substrate preferences and physiochemical properties. The study also demonstrated the importance of esterases from various sources and the advantages of metagenomic screening as it introduces novel genes. These genes can have many industrial applications. Esterases are also highly important as revealed in this study by their use in the synthesis of optically pure compounds that can be used for medicinal purposes.

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